ELECTROENCEPHALOGRAPHIC (EEG) SEIZURES AND BACKGROUND ABNORMALITIES IN A RAT MODEL OF NEONATAL HYPOXIC-ISCHEMIC ENCEPHALOPATHY

by

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ABSTRACT

An important clinical problem that has not been adequately addressed in animal models is the rapid and reliable detection of cerebral dysfunction and brain damage. The use of continuous electroencephalographic (EEG) monitoring has the potential to address this unmet medical need, but for technical reasons translational research with animal models of neonatal hypoxic-ischemic encephalopathy (HIE) has lagged behind clinical research on human neonates in this area. Previously, large animal models, such as sheep/lambs were required to study the quantitative features of EEG in animal models of neonatal brain injury. In this study, we (1) developed and adapted a miniature wireless EEG system for use in rat pups as young as postnatal day 6 (P6); (2) compared seizures and EEG background in two animal models of acute neonatal seizures (hypoxia with seizures but no obvious neuronal death; and, hypoxia-ischemia with seizures and catastrophic brain damage); and (3) quantitatively analyzed electrographic seizures and background EEG abnormalities during the subacute period in these rat-pup models (i.e., hours to days *after* hypoxia alone and HIE). We showed that the miniature telemetry system allowed repeated recordings of electrographic activity during and at different times after the insult in individual animals; these recordings had high signal-to-noise ratio and low number of artifacts, which in turn allowed quantitative analyses of both the electrographic seizures and

changes in the background EEG. The recordings *during* the two insults (i.e., hypoxia alone and HIE) showed that the acute hypoxic environment was the driving force for the electrographic seizures in both models, but that *brain damage* was associated with a progressive suppression of both the ongoing seizures and the background EEG. During the subacute period, however, rat pups that had experienced hypoxia alone showed no seizures and displayed a background EEG virtually identical to sham-control animals. The rat pups with a catastrophic lesion and HIE consistently had suppression of the background EEG (and some showed electrographic seizures during the intermittent recordings). Most important, quantitative analyses with Fast Fourier Transforms from brief periods of EEG recording (i.e., as short as 1 min) could rapidly and reliably detect those animals that were experiencing HIE. Our findings may translate to the human neonatal population at risk for HIE.

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CHAPTER 1

INTRODUCTION

Brain injuries before and during delivery are a major cause of neurologic insults that can be associated with serious sequelae. Prospective prediction of long-term outcome in neonates that have suffered a stroke or other insults that result in hypoxic-ischemic encephalopathies (HIE) is difficult. Current strategies rely on a clinical exam, occurrence of neonatal seizures and imaging. A report of *clinical seizures* (i.e., behavioral seizures or convulsions) often triggers further evaluation for stroke or other underlying abnormality (Nelson and Lynch, 2004). Not all seizures are accompanied by a behavioral component, and many children with a neurologic insult may not be evaluated and treated soon enough to result in the most effective intervention. Seizures do not necessarily lead to a negative outcome; some children that have suffered through multiple seizures recover and appear completely normal later in life (Mercuri et al., 1999). Thus, an ability to identify rapidly the underlying cause of the seizures would be extremely beneficial. Neurologic insults do not always result in an immediate and obvious abnormality. Imaging techniques are highly effective at detection of the anatomically apparent abnormalities by providing excellent spatial localization. Imaging is generally performed as a part of diagnostic analysis for infants with

behavioral seizures, but this approach generally reveals the underlying insult retrospectively. Hypothermia, a therapy that has been reported to be most effective in improving outcomes of HIEs and strokes, has a window of optimal successful outcome of 6 h after the insult (Khurshid et al., 2011; Thoresen et al., 1995; Gunn et al., 1998; Azzorardi et al., 2009). Clearly, a simple technique with good temporal resolution that would allow minute-by-minute assessment of cerebral activity and facilitate the screening of neonates for application of hypothermia would be extremely beneficial. The purpose of this study was to identify electrographic features of the neonatal EEG that are associated with – or potentially predictive of - negative outcomes; this was accomplished with the use of two related animal models (perinatal hypoxia alone [Ha] versus hypoxia-ischemia [HI]), where the etiology and outcomes can be manipulated experimentally.

Hypoxic-ischemic injuries

Hypoxic-ischemic encephalopathy (HIE) is a serious complication in the neonatal population. The condition is often caused by asphyxia before and/or during birth. The incidence of asphyxia is 2-4 per 1000 full-term infants and is nearly 60% in premature low birth-weight newborns (MacDonald et al., 1980). Between 20-50% of infants with HIE die while they are newborns, and asphyxia-caused complications account for 23% of all newborn deaths globally (Lawn at al., 2005; Vannucci et al., 1999). Of those that survive, 25% will go on to develop major permanent neurologic abnormalities and handicaps such as cerebral palsy,

epilepsy and intellectual developmental disabilities (Vannucci et al., 1999). These conditions often cause chronic impairment and require long-term supportive care and services. Cerebral palsy has been estimated at $11.5 billion lifetime costs and intellectual and developmental disabilities at a cost of $51.2 billion (in 2003 dollars) for persons that were born in 2000. Annual costs of management of epilepsy have been estimated to be $15.5 billion per year (CDC, 2007). While HIE is not the only cause or etiology of these conditions, it remains an important contributor to these injuries and the resulting costs. Development of new and highly effective treatments for HIE has the potential to reduce these enormous human and economic costs.

HIE etiology

HIE is primarily caused by the combination of two main factors: hypoxemia (abnormally low partial pressure of oxygen in the blood) and ischemia (lack of blood flow into the brain, or part of the brain). In the intra-partum period (i.e. during the delivery), hypoxemia and ischemia can interact causing a hypoxic-ischemic (HI) injury to the brain. During intra-partum asphyxia, hypoxemia can directly cause cardiac insufficiency and loss of cerebrovascular regulation, which results in an ischemic insult (Volpe, 2001). Other antepartum and intra-partum etiologies - such as placental abnormalities, maternal hypotension, maternal diabetes, preeclampsia, birth trauma, prolonged labor, umbilical cord disturbances, fetal heart abnormalities, infections, sepsis, severe under-nutrition and postnatal insults often associated with premature birth - can cause HI brain

injuries (Volpe, 2001; Hill and Volpe, 1982). While perinatal insults were thought to be uncommon (10%) in the development of HIE, other evidence suggests that the actual percentage may be much higher (Cowan et al., 2003). The etiologies of HIE are highly variable; however, they can *all* result in a devastating neurologic injury that leads to long-term negative outcomes. Strategies for early detection and reduction of the HIE-related cerebral injuries of various etiologies are an obvious target for research in animal models, where the underlying cause of the insult can be manipulated.

Neonatal seizures – clinical prevalence and etiology

Seizures in neonates are often the first warning signs of HIE and other ischemic insults. Neonatal seizures are seizures that occur in the first month of the infant's life. The risk of neonatal seizures has been reported to be 2.84-3.5 per 1000 live births with a much higher risk (57.5 per 1000) in premature and low birth-weight neonates of <1500 g (Lanska et al., 1995; Lanska and Lanska, 1996). Clinically, neonatal seizures are considered to be a signal of an underlying neurologic or physiologic abnormality. Seizures can be caused by several etiologies such as HIE, intracranial hemorrhage, developmental defects, metabolic disturbances, intoxication by local anesthetics and drug withdrawal (Volpe, 2001). Whether the seizures are a direct cause, or merely the result, of another insult remains controversial. Most clinicians agree that neonatal seizures should be treated; they are often caused by an underlying illness and can

interfere with supportive measures (such as assisted respiration) used to treat the underlying problem. Some seizure etiologies, such as late onset hypocalcemia and subarachnoid hemorrhage, can resolve and have favorable outcomes (>90%) (Volpe, 2001). Treating seizures, however, carries a risk; overly aggressive treatment with anti-seizure medications may cause abnormal development and low IQ scores in humans and may inhibit neurogenesis, induce apoptosis and disrupt striatal development in animal models (Camfield, 1997; Bourgeois et al., 1983; Forcelli et al., 2012; Forcelli et al., 2011; Chen et al., 2009; Stefovska et al., 2008; Bhardwaj et al., 2012). Currently, it is not clear whether neonatal seizures *per se* cause brain injuries, but it has been reported that seizures can aggravate the underlying infarct by causing a secondary injury or triggering additional cell death (Wirrell et al., 2001; Williams et al., 1992; Aso et al., 1990). Thus, clinicians normally treat all cases of confirmed neonatal seizures. An important area of research that could be valuable in the clinic (but that is currently lacking in the animal models) is the development of strategies to rapidly differentiate between harmful and benign etiologies.

Do seizures *per se* damage neonatal brain: insights from animal models

Literature where animal models are utilized to study neonatal seizures, brain damage and subsequent development of epilepsy does not provide a clear answer to whether seizures *cause* damage in the neonatal brain and whether the

seizures can *directly* cause epilepsy. Several studies reported that in a rat model of neonatal seizures, kainate-induced status epilepticus was not sufficient to induce neuronal death in the hippocampus (Sperber et al., 1991; Nitecka et al., 1984). Additionally, it was reported that when rat pups were treated with kainate between P5 and P10, no development of subsequent epilepsy was reported and latency to flurothyl-induced seizures was not altered (Staftstrom et al., 1992). A possible mechanism that was proposed as to why neonatal seizures *per se* do not cause neuronal death is age-dependent properties of GluR2 glutamate receptor subunit (Moshe et al., 1998). In adult rats, seizures cause a down-regulation of GluR2 subunit, allowing more Ca^{2+} to enter the cell, thus triggering cell death; in the immature brain, this down-regulation is thought to be absent, resulting in less Ca^{2+} entering the neurons, thus providing a possible mechanism of neuroprotection (Friedman et al., 1994; 1997). Due to the difficulty of using immature rat pups for recording of EEG, with few exceptions (Hirsch et al., 1992), no quantitative studies that examine neonatal seizure properties have been conducted to date using rodent models. It is common that in animal models of this age, EEG is used to verify the presence of behavioral seizures, but electrographic seizure activity has not been examined quantitatively. A quantitative analysis of seizures has the potential to give insights that would let us differentiate between harmful and benign seizures.

EEG: significance of seizures and background patterns

In the epilepsy field, research has been focused mostly on seizures, their effects on the brain, and their treatment. While epilepsy is one of the possible outcomes of HIE, and acute seizures have been demonstrated to have a role in injuring the brain, an extremely important clinical factor is the concept of *background EEG* activity (Wirrell et al., 2001; Williams et al., 1992; Aso et al., 1990). In adults, the EEG is mostly used as a seizure detector or to confirm brain death; however, in neonates and infants, examination of the EEG as a whole (seizures and background) has been reported to predict outcomes retrospectively. Background EEG is particularly useful in the prognosis of outcome in neonates with seizures. Background EEG abnormalities of amplitude, frequency, continuity, symmetry, sleep states and maturation have all been described to have prognostic value (Holmes and Lombroso, 1993; Clancy and Legido, 1991; Tharp et al, 1989; Legido et al., 1991; Shinnar et al., 1990; Pezzani et al., 1986; Korotchikova et al., 2011; Tharp et al., 1981; Monod et al., 1972). In cases where seizures occurred on an otherwise normal EEG background, the outcome was reported to be positive (<10% with neurological sequelae); however, if neonatal seizures were recorded on an abnormal background, >90% of outcomes were severely negative (Volpe, 2001; Rowe et al., 1985). Additionally, seizures with severe background abnormalities in the EEG were highly correlated with being refractory to AED therapies (Connell et al., 1989). A proof-of-concept of detecting background abnormalities with

quantitative EEG (qEEG) has been established. Korotchikova and colleagues (2011) developed a qEEG analysis technique that is predictive of the grade of HIE based on background EEG activity. Therefore, studies that extend beyond seizures in the EEG analysis may provide valuable insights for predicting outcomes and applying therapies for neurologic disorders. In animal models of neonatal neurologic conditions, such as HIE, background EEG has not been widely used. Several studies in immature sheep and piglets have examined the effect of HIE on EEG background (Williams et al., 1992; Bjorkman et al., 2010); however, these techniques have not been used in rodent animal models, where the focus has remained largely on behavioral seizures only.

Acute neonatal seizures, brain damage and acquired epileptogenesis

The relationship between acute neonatal seizures and acquired epileptogenesis is currently not clear. Controversy exists on whether neuronal death is required for development of epilepsy. Several groups have reported that epileptogenesis and subsequent spontaneous recurrent seizures may occur after acute seizures that result in no histologically detectable brain damage (i.e. negative outcome present, but independent of brain damage caused by the seizures) induced by pilocarpine, hypoxia or hyperthermia (Raol et al., 2003; Jensen et al., 1991; Dube et al., 2010; Rakhade et al., 2011). However, previous studies in our laboratory using an animal model of neonatal HIE showed that

while all animals that were subjected to HIE had acute behavioral neonatal seizures, only those with an *overt brain lesion* went on to develop epilepsy (Kadam et al., 2007, 2011). Another study showed spontaneous epileptic seizures in in a model of radiation-induced cortical dysplasia where lesions were induced before birth and EEG was monitored as adults (Kondo et al., 2001). Clinical evidence suggests that development of epilepsy is very common in children with cerebral palsy, a condition that is consistently associated with an overt brain injury (Cummins et al., 1993; Aneja et al., 2001; Lee et al., 2005). Several other seizure presentations in neonates and children result in no overt brain injury. Febrile seizures in children have been reported to not be associated with a negative long-term outcome (Maytal et al., 1989; Nelson and Ellenberg, 1976; Berg and Shinnar, 1991). In fact, it is common practice in many institutions to not treat simple febrile seizures with anti-epileptic drug therapies. In the study of Kadam et al. (2011), all animals were subjected to HI, but only half of them developed lesions and subsequent epilepsy. Unpublished data from our laboratory as well as others (Jensen et al., 1991; Rakhade et al., 2011) suggests that subjecting rat pups to hypoxia, which is a part of the experimental HI-induction protocol, causes acute *behavioral* neonatal seizures during the hypoxia. Thus, it is highly probable that while only half of the animals that developed lesions in the Kadam et al., (2011) study, all of them were subjected to hypoxia-induced *behavioral* convulsions. However, it is not clear whether the acute seizures were quantitatively similar or different in the animals that did or did not have cerebral lesions. Recording EEG and quantitatively analyzing

seizures in animals with and without brain damage could provide us with a valuable answer regarding the significance of acute neonatal seizures in the process of acquired epileptogenesis.

Clinical (behavioral) and electrographic seizures

Seizures are usually first identified using their behavioral (clinical) component. In adults, epileptic seizures can be identified by clinical exam, self-report and altered states of consciousness. In neonates, the clinical exams are more difficult to perform and seizure manifestations are often subtle, thus a seizure diagnosis is sometimes missed (Connell et al., 1989; Wusthoff et al., 2011; Levene, 1993; Mercuri et al., 1999). Additionally, neonatal seizures often have no behavioral manifestation (Boylan et al., 2002; Painter et al., 1999; Connell et al., 1989). This phenomenon is called electro-clinical decoupling. It is often exacerbated by administration of therapies such as phenobarbital - a GABA-A agonist, the current standard-of-care for neonatal seizures. Several studies have reported that phenobarbital often suppresses clinical seizures, but has no effect on electrographic EEG activity (Clancy et al., 1988; Connell et al., 1989; Scher et al., 2003). Electro-clinical decoupling has the dangerous potential to make an otherwise sick infant appear normal, thus continuous EEG monitoring is essential in such cases.

Treatment of neonatal seizures

Few if any drugs are designed specifically for treatment of neonatal seizures. The current standard-of-care, phenobarbital, has been on the market since 1912, is sometimes ineffective in as many as 43-70% of the infants, and often causes electro-clinical decoupling (Gillman et al., 1989; Gal et al., 1984; Connell et al., 1989; Clancy et al., 1988). A notable strategy aimed at exploiting the age-dependent intracellular chloride homeostasis is the use of bumetanide, a diuretic compound that blocks the NKCC1 chloride transporter in the immature brain. This approach has been reported to be effective in attenuating abnormal activity in brain slices and reducing EEG power of kainate-induced seizures (Dzhala et al., 2005; Rheims et al., 2008; Kilb et al., 2007). Recently, however, the safety and efficacy of this approach has been questioned (Vanhatalo et al., 2009; Chabwine and Vanden Eijnden, 2011), so the search for new highly effective compounds to treat neonatal seizures remains an open-ended challenge. Animal models are routinely used for drug discovery. In the studies that utilize these models, it is common that the ability to attenuate behavioral seizures is used as a measure of efficacy for testing of new therapeutic approaches (Aujla et al., 2009; Koh et al., 2004; Lai et al., 2009; Mikati et al., 2007; Koh and Jensen, 2001; Folbergrova, 1997, 1994). Using attenuation of clinical seizures as the measure of efficacy excludes the possible effects on electrographic seizures and electro-clinical decoupling. Recording EEG from immature rats of a developmentally appropriate age (P6-12) has been challenging because of a lack of effective instrumentation, difficult surgical

implantation of recording devices, and the rat's innate maternal care that is incompatible with the majority of EEG devices on the market. Development of devices and strategies that would allow EEG monitoring and quantitative analysis techniques would greatly improve drug discovery and the drug-testing process.

Research goals

The goal of the current research is to develop an EEG recording technique usable on immature rat pups as young as P6 that would allow for simple quantitative analyses of EEG signals. The requirements for the technique include: (1) good signal-to-noise ratio, (2) uncomplicated and quick surgical implantation, and (3) the ability to conduct serial recordings in pups reared by the dam. Development of such a technique would enable us to address several important hypotheses that have not been investigated in rodent animal models due to lack of instrumentation.

Having developed an EEG recording technique that is feasible in rat pups, we compared electrographic activity in two models of acute neonatal seizures – hypoxia alone (Ha) and hypoxia-ischemia (HI). In the Ha model of acute neonatal seizures, the pups were exposed to 8% hypoxia for 2 h, which induces acute seizures with no neuronal degeneration. In the HI model, the rat pups were also exposed to 8% hypoxia, but in addition their right carotid artery was ligated. This model results in a lesion similar to that in humans with HIE (Kadam et al., 2007; 2011; Vannucci et al., 1998; Levine, 1960). First, we hypothesized that due to a severe brain injury in the HI-treated animals, they would have more intense

seizure activity when examined quantitatively. Second, we hypothesized that both HI- and Ha-treated animals would have seizures and unique quantifiable abnormalities in the background EEG in the subacute period. To test these hypotheses, considerable development and testing of a miniature telemetry system was first undertaken (first manuscript). The two hypotheses – and others - were then tested in a series of experiments during (second manuscript) and after perinatal Ha/HI (third manuscript).

CHAPTER 2

RECORDING EEG IN IMMATURE RATS WITH A NOVEL MINIATURE TELEMETRY SYSTEM

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Abstract

Serial electroencephalograpic (EEG) recordings from immature rat pups are extremely difficult, but essential for analyzing animal models of neonatal seizures and other pediatric neurologic conditions. In this report, we describe the features and applications of a novel miniature telemetry system designed to record EEG in rat pups as young as postnatal day 6 (P6). First, we have utilized serial EEG recordings to record age-dependent changes in the background EEG signal as the animals matured from P7 to P11. Second, we have recorded electrographic seizure activity in two animal models of neonatal seizures, hypoxia- and kainate-induced seizures at P7. Third, we describe a viable approach for long-term continuous EEG monitoring of naturally-reared rat pups implanted with EEG at P6. The important advantages of using miniature wireless EEG technology are (1) minimally invasive surgical implantation; (2) a device form-factor that is compatible with housing of rat pups with the dam and littermates; (3) serial recordings of EEG activity and (4) low power consumption of the unit, allowing continuous monitoring for up to 2 years without surgical re-implantation. The miniature EEG telemetry system provides a technical advance that allows researchers to record continuous and serial EEG recordings in neonatal rodent models of human neurological disorders, study the progression of the disease, and then assess possible therapies using quantitative EEG as an outcome measure. This new technical approach will enable us to improve animal modeling of human conditions that rely on EEG monitoring for diagnosis and therapy.

Introduction

EEG in the clinic and animal models

Electroencephalographic (EEG) recordings are an essential test for clinical diagnosis and outcome prediction of various neurologic conditions and injuries. EEG is required to detect nonconvulsive seizures, helping to change the clinical treatment of neonatal and pediatric patients (Abend et al., 2011; Nash et al., 2011; Connell et al., 1989; Glass et al., 2009;). EEG recordings have been highly effective for prediction of the outcomes of neurologic insults such as perinatal hypoxia-ischemia (HI) and asphyxia (Korotchikova et al., 2011; Holmes and Lombroso, 1991; Aso et al., 1990; Murray et al., 2009; Hellstrom-Westras and Rosen, 2005; Garfinkle and Shevell, 2010; Walsh et al., 2011). EEG has been used with a high degree of success for neonatal and pediatric seizures and brain injuries; however, it has been underutilized in animal models. Rodent animal models are an important tool for preclinical testing of anticonvulsant compounds that are used for treatment of epilepsy. In order to improve overall validity of neonatal animal models of human disorders, it is critical to use methods and techniques similar to those that are used to diagnose and manage human patients in the clinic. To address these issues, we describe here a wireless EEG telemetry system that is highly effective in immature rodents.

EEG in immature animals

Obtaining high-quality, serial EEG recordings from immature rodents as young as P6 is extremely difficult and has been largely unsuccessful. Most of the

previous studies examining in vivo EEG or local field potential (LFP) recordings in immature rats have been conducted using wired recording solutions in animals that were postnatal day 12 (P12) or older (Cyacong et al., 2011). Several published studies suggest that the P7-P12 rat pup is the developmental age that corresponds to a full-term human neonate (Quinn, 2005; Romjin et al., 1991). However, the largest burden from neonatal seizures and other neurological abnormalities exists in the premature infant population (Cummins et al., 1993). This raises the importance of modeling the disorders in rat pups at a younger age (i.e., P6-P9). Working with rat pups that are younger than P12 is more difficult, and requires specialized surgical, recording and rearing strategies. To evaluate an animal model, we need to quantitatively analyze the entire disease process, including the acute period, progression and outcome; thus, serial recordings from the same animals are critical for translational analyses. Making serial EEG recordings would not only enable the ability to examine the acute period, but also would allow us to quantitatively evaluate progression of the disease after an injury or following an intervention. Accomplishing the goal of making serial recordings starting at P6 required several unique technical innovations and solutions.

Significance of wireless EEG in immature rodents

Here we describe design features of a miniature EEG telemetry system and surgical techniques that make it compatible with use in immature rat pups as young as P6. We use two models of neonatal seizures in P7 rat pups to record

electrographic seizures, and describe age-dependent features of the normal EEG. Additionally, we show long-term monitoring approaches that enable continuous serial monitoring of animals from pup to adult ages. We show that animal models of neurologic conditions do not have to be limited to behavioral outcome measures; instead, EEG can be used for longitudinal, quantitative electrophysiological analysis.

Materials and methods

Wireless transmitter and receiver

The requirements for making EEG recordings in rat pups dictate that the device be small, have a low profile, and have minimal power requirements. To accomplish the low-power and small form-factor demands, we used the following design. The device consists of two fundamental components: (1) a micro-transmitter comprised of a physiological amplifier controlling a pulse-width (i.e. frequency) modulation oscillator, and (2) a capacitive-coupled receiver, which includes a frequency-to-voltage converter that recovers the original EEG signal. The recording input is two leads connected to an amplifier. The amplitude of the EEG signal modulates the pulse width of a square-wave oscillator, which is transmitted via capacitive coupling to the antenna. A high-impedance receiver then detects, amplifies, and filters the EEG signal. The receiver consists of an integrator (or a frequency-to-voltage converter) and a band-pass filter, which recovers the original AC signal from the transmitter. The bandwidth of the device

is 0.1 – 120 Hz, which is suitable for many experiments recording EEG signals for long-term monitoring.

Device form factor

Form factor is an important consideration in a device designed for use in immature rats (Figure 2.1). The implanted device needs to be stabilized without the use of skull screws. Stability and durability are critical because rat pups younger than P21 are housed and reared with their littermates and the dam, who will remove extraneous objects from pups. To achieve the required durability, the transmitter is encased in optically clear epoxy (Epo-tek 301; Billerica, MA). The mold was designed in the shape of a cylinder that is 10 mm diameter and 10 mm high, with a dome-shaped top (Figure 2.2). This shape was the most dam-resistant and was easy to affix to the top of the rat-pup skull. The bottom of the transmitter that comes in contact with the skull is slightly concave, which increases the contact area with the slightly convex rat skull. The increased contact area greatly improves the effectiveness of fixation by cyanoacrylate glue, eliminating the need for skull screws.

Animals

All surgical procedures were performed under protocols approved by the University of Utah Animal Care and Use Committee. Pregnant Sprague-Dawley adult female rats (14 days gestation) were received from Charles-River (Wilmington, MA). Pups were delivered in the animal facility approximately 1

week after the arrival of the pregnant female (University of Utah, Salt Lake City, UT). The litter size varied from 8 to 10 pups. Animals were housed with the dam and littermates, and at P6 they were implanted with the transmitter of the miniature telemetry system.

Surgical implantation of the transmitter unit

During the surgical procedure, animals were initially anesthetized with 4% isoflurane (MWI Veterinary Supply, Meridian, ID) and maintained at 2% during the procedure. Surgical equipment and ear bars were autoclaved and stereotaxic frame was sprayed with 70% ethanol. Sterility of surgical tools was maintained with 70% ethanol. Rat pups were stabilized in the stereotaxic frame with ear bars designed for small animals (David Kopf Instruments). An incision was made across the midline of the scalp using a scalpel (no. 15, Bard-Parker Safety Lock, Becton Dickinson and Company, Franklin Lakes, NJ). After the incision was made, the skin was pulled aside and clamped with hemostats to ensure access to the surgical field on the top of the skull (Figure 2.3A). The periosteum was removed using sterile cotton swabs (Puritan Medical Products Company, Guilford, ME) and areas of surface bleeding were cauterized with a fine-tip, low-temperature cautery pen (Bovie Medical, Clearwater, FL). Two electrode holes were drilled using a dental drill with a 0.7mm burr (Fine Science Tools), 2 mm lateral from midline of the skull, separated by 2 mm, anterior-posterior (Figure 2.3B). The transmitter electrode wires were trimmed to the length corresponding to the depth of the dura (Figure 2.3C) and were inserted into the burr holes. The

unit was then fixed on the surface of the skull using cyanoacrylate gel compound (Loctite 454) with accelerator (Loctite 7452). Additional cyanoacrylate was applied around the unit and the exposed areas of the skull to stabilize the implant (Figure 2.3D). The area was rinsed with warm sterile saline and the skin was sutured with Vicryl 4-0 coated polyglactin 910 suture (Ethicon) (Figure 2.3E). Anesthesia was terminated and the animals were injected with 0.5 mL of lactated Ringers (sub-cutaneous), and superficially treated with 0.5 mL local anesthetic (Marcaine) (Figure 2.3F). The entire surgical procedure was kept to <10 min. Pups were then allowed to recover with the dam and littermates for 24 h prior to treatment.

Hypoxia recording chamber

Hypoxia in P7 rat pups was induced in a specially designed hypoxia chamber with integrated telemetry receiver bases (Figure 2.4). The chamber was designed with a clear acrylic housing (65 x 33 x 41 cm), a hinged lid and a stable aluminum platform where the capacitive-coupled receiver “bases” were placed. Each chamber could house up to three receiver bases. A feedback-controlled proportion-integrate-derivative (PID) temperature controller unit with thermocouple regulated the temperature inside of the chamber. Temperature was held at 37°C for the duration of recordings. The PID controller modulated a fan with heating elements located below the platform. The fan circulated warm air in the chamber by unidirectional flow. Chamber temperature was additionally verified by an independent thermocouple and logged to the recording computer

(Vernier Instruments, Beaverton, OR). Each animal was placed in a sealed acrylic treatment chamber that rests on a receiver base, and gas mixture was administered at a positive pressure rate of 1 L/min through a manifold. The design of the recording chamber allowed for parallel recordings of multiple animals. Because each chamber had separate gas inputs under positive pressure, multiple gas mixtures could be used within each of the housings. EEG signals from multiple receivers were digitized by an analog-to-digital converter (Biopac MP150, Biopac Systems, Goleta, CA), sampled at 500 Hz and stored on a computer using Acknowledge 4.1.1 software (Biopac, Goleta, CA).

Hypoxia induction protocol

Hypoxia was induced in rat pups at P7 after recovery from implantation of the device at P6. Animals were placed in the recording chamber with normoxic air, and baseline activity was recorded for 30 min. After the baseline recording, a hypoxia mixture of 8% oxygen, 92% nitrogen was introduced into the chamber. The duration of hypoxia administration was 2 h. EEG was recorded continuously during the time when animals were hypoxic. After treatment, the pups were given 0.5 mL of lactated Ringer's solution subcutaneously. Animals were then returned to the dam and allowed to recover.

Kainate induction protocol

For the kainate-induced seizure model, P7-8 rat pups were used. The treatment and recordings were conducted in the previously described chamber

with normoxic air and chamber temperature held at 37 °C. Baseline EEG was recorded for 30 min. Kainate was dissolved in sterile saline and injected intraperitoneal (IP) at 2 mg/kg. Another dose of 1 mg/kg was administered after 40 min. EEG was recorded for 3 h after the first administration of kainate. Pups were then given 0.5 mL of lactated Ringer's solution subcutaneously and were allowed to recover with the dam and littermates.

Long-term recording protocol

Two methods were used for long-term recordings. For the first method, animals were monitored in 2 h epochs every day from P7 until P11 in the previously described recording chamber. For the second method, one rat pup implanted with the wireless transmitter was placed in a cage with its dam and littermates. The cage was then placed on an adult-sized EEG receiver that allows for 24-h per day monitoring in the care of the dam.

Quantitative EEG analysis

For each animal, 30 min of EEG data were selected from each day of the recording. Signal dropout artifacts were manually removed from the EEG. The data files were then converted into MATLAB format (Mathworks, Natick, MA). Fast Fourier Transforms (FFTs) were performed to analyze EEG data in the frequency domain from 0 to 60 Hz. Power spectral densities (PSDs) were estimated from the FFT using 2048 Hann-window segments based on the Welch method and normalized by $10*\log_{10}$ (PSD). Power levels at all frequencies in 0.1

to 60 Hz were plotted with 95% confidence intervals. To compute integrated EEG power, the area under the FFT curve was integrated in the frequency ranges defined by EEG bands: delta 0.1-4 Hz, theta 4-8 Hz, alpha 8-13 Hz, beta 13-30 Hz, gamma 30-60 Hz (Krauss and Fisher, 2006; Stockard-Pope et al., 1992).

Results

Neonatal seizures in hypoxia- and kainate-induced models

The telemetry device allowed us to record EEG activity from two different models of neonatal seizures. Seizures were induced by lowering the concentration of oxygen to 8% (i.e., hypoxia, n = 12) or by administering a chemo-convulsant compound, kainate (n = 5). These models presented with different types of seizures (Figure 2.5). Hypoxia-induced seizures had characteristic EEG patterns that began immediately after introduction of the hypoxia gas mixture into the treatment chamber (Figure 2.5A). The EEG discharges during 2 h of hypoxic treatment included high-amplitude, low-frequency bursts, which were accompanied by classic tonic-clonic convulsive behaviors. Additionally, lower-amplitude, higher-frequency discharges were present, accompanied with a “shiver-like” behavior of the animal with no classic convulsive features. Inter-ictal, short, high-amplitude discharges were present between convulsions, but did not show a specific behavior at the time of the discharge. The behaviors and abnormal EEG patterns ceased upon introduction of normoxic environment in the treatment chamber. The behavioral and EEG findings were similar in all of the treated animals (12/12). Kainate elicited a

different EEG and behavioral pattern in rat pups. Seizures began 15-30 min after an injection of a 2-mg/kg dose of kainate. The EEG began with high-frequency discharges that continued to be abnormal for up to 4 h after the first seizure (Figure 2.5B). All animals injected with kainate showed similar discharge patterns. Clinical correlates of the seizure activity detected on EEG included myoclonic jerks, tonic stiffening, limb clonus and behavioral arrest. Behavioral and electrographic seizures were present in 5/5 animals that were injected with kainate. Additionally, the kainate-induced seizure activity was detectable at P15 (Figure 2.6) when electrodes were implanted with no burr holes at P7.

Age-dependent features of the background EEG

The recording electrode configuration enabled us to record not only seizures, but also background (e.g., normal) EEG patterns with excellent signal-to-noise quality. We used Fast Fourier Transforms (FFT) to quantify the power of classically defined EEG bands (delta: 0.1 – 4 Hz, theta: 4-8 Hz, alpha: 8-13 Hz, beta: 13-30 Hz, gamma: 30-60 Hz) as the animals matured from P7 to P11 (n=10, serial recordings). The characteristic pattern of activity in these EEG bands included diffuse patterns in the signal without discrete oscillations. When examined visually, the EEG signal had qualitative changes as the animals matured. These changes could be quantified using FFT (Figure 2.7). At lower frequency bands (i.e., delta, theta, alpha), power increased from P7 to P8 and stabilized from P8 to P11 (Figure 2.7). In the beta and gamma bands, power showed a gradual increase from P7 to P10, with stabilization occurring between

P10 to P11 (Figure 2.7). Age-dependent changes in the signal were detected in all of the EEG bands by integrating the power under the FFT curve in each of the bands and finding the mean of integrated power between animals of each age group. The profile of integrated power follows the above-described FFT profile (Figure 2.8; Table 2.1).

Long-term monitoring

In order to study models of epilepsy and other spontaneous EEG events, 24-h continuous monitoring is necessary. However, these experiments are difficult because the rat pup is absolutely dependent on the dam for survival and normal development. For a proof-of-concept experiment for 24 h monitoring, we implanted two rat pups with the EEG telemetry at P6 and housed them with the dam and littermates. The animals were then placed on a receiver base designed for an adult animal under standard animal housing conditions. We then recorded EEG in a rat pup for a period of 48 h continuously (Figure 2.9). Several EEG signal “dropouts” were present, but overall signal quality was excellent and enough data was collected to be able to detect and ultimately quantitate EEG abnormalities. None of the animals had spontaneous seizures in the signal. Over the course of the experiment, the other animals that were housed in the same cage did not damage the implant. Overall, electrical and movement artifacts recorded with the telemetry system were minimal. Compared with wired recordings, artifacts recorded with the telemetry system were much shorter duration and were less frequent (Figure 2.10B). Instances where the receiver

antenna did not properly couple with the transmitter on the animal's skull resulted in a "drop out" artifact where the EEG maintains a zero potential until the signal was detected again (Figure 2.10C).

Discussion

Telemetry vs. wired recording techniques

Wired systems can be used in freely behaving animals, but the signal is extremely susceptible to movement artifacts (Figure 2.10A). Skull bones in immature rodents are flexible and the tether forces act like a lever, amplifying the bending of the electrodes relative to the skull as the animal moves around the cage. These forces cause flexing of the bones in the skull, resulting in major movement-related electrical artifacts. The presence of these artifacts in the signal makes analysis more difficult and obscures the EEG signals that normally occur when the animal is freely moving around the cage. Movement artifacts preclude the use of automated techniques for high-throughput analysis of the EEG data. This wireless system solves this problem by removing the need for tethering. The implant is light and stabilized on the surface of the skull, greatly reducing movement artifacts in the EEG (Figure 2.10B). The telemetry system is susceptible to signal drop out artifacts where the electric field generated by the transmitter is smaller than the receiver antenna is able to detect. These occur when the animal is positioned sideways with the transmitter antenna parallel, or 90° out of phase, to the receiver antenna. Other artifacts can occur when the animal contacts the metal water spout, or when the animal comes in contact with

the metal wire-top of the cage. In order to have minimal impact on the signal, we designed the receiver base to cancel these artifacts by clamping the signal to 0 V when the contact between the antennae is lost. Unlike movement artifacts in the wired system, which present as multifrequency high-amplitude bursts that can saturate the signal and make quantitative frequency analysis difficult, the drop out artifacts have minimal impact on the frequencies in the EEG signal. However, during the drop out artifacts, the EEG signal is not detectable, making a false-negative result possible if an animal had a seizure or abnormal EEG discharge during the artifact. This is unlikely because convulsions are often associated with robust movement, making the probability that the animal will remain in a position with signal “drop out” low over the course of the seizure. These signal “drop outs” tend to be limited to a few milliseconds. Reduction of electrical artifacts and hardware-enabled strategies that would reduce their impact on the quality of the EEG signal is a substantial advantage of the miniature telemetry system.

Surgical procedures

The small, self-contained form factor of the package and reduced requirements for reinforcing the implant to the skull enabled us to design surgical procedures that are less invasive and require shorter periods of anesthesia. Tethered systems are usually stabilized on the surface of the skull using multiple stainless-steel skull screws and dental cement (Ekstrand et al., 2011; Lehmkuhle et al., 2009). Implantation of the telemetry unit requires two burr holes for electrodes and a small amount of cyanoacrylate gel glue that binds the implant to

the bones of the skull, in contrast to most wired implants that require three or more burr holes for skull screws, in addition to the burr holes for the electrodes. The wireless telemetry system can be implanted in 10-15 min, compared to 45 min to 1 h required to implant a typical wired unit. Less-invasive, shorter surgeries improve animal survival and recovery time. One of the traumatic procedures with most EEG recording techniques is the surgical implantation of the electrode wires into the brain. The wires and surgery cause trauma from the procedure itself that could confound the EEG signal. The trauma includes bleeding, disruption of the blood-brain barrier, and increased possibilities of infection by compromising the integrity of the skull. In order to implant the EEG electrodes, a skull burr hole is normally used. However, classical EEG in humans is a noninvasive technique where electrodes are placed on the scalp. In an attempt to reduce the trauma of making burr holes in the skull and placing electrodes through the holes, we tested a hole-free implantation method by placing electrodes on the surface of the skull without burr holes. A rat pup was implanted with this method at P6, and EEG signal quality was verified by inducing status epilepticus with kainate at P15 (Figure 2.6).

Size and power requirements

The telemetry system described here is unique; however, other telemetry systems are currently available for research use. A long-standing problem with telemetry systems in general is the size of the transmitter and the method of implantation. Many radio-frequency based systems require intraperitoneal

implantation of the transmitter or require the use of a “backpack” system. This feature has several advantages, but is not suitable for use in P6-P7 animals due to space limitations, size and weight of the transmitter, and the animal’s dependence upon the dam for survival. Thus, most physiological and/or translational research on neonatal animals has used large animals, such as pigs, dogs or goats (Bjorkman et al., 2010; Williams et al., 1992; Sherman et al., 1999). Power requirements are another inherent disadvantage of telemetry systems. While wired systems are inherently passive, telemetry requires the use of batteries, a power source, or a transducer to power the transmitter. This limits the useful life of most transmitters to 6-12 months, or even hours/days/weeks in some cases. The telemetry system described here uses capacitive coupling for transmission of the signal, which limits the power draw to 8 mA at 1.5 V. The low power draw enables the useful life of the transmitter to be up to 24 months from a single #303 silver-oxide battery, theoretically allowing continuous monitoring from an immature age until death in most rodents.

Single channel per animal per cage

The current iteration of the miniature telemetry system design allows for recording of one channel of EEG in one animal per receiver base. This design limitation requires single housing of the animals or housing one animal implanted with the transmitter per cage. Studies that require chronic 24-h monitoring of EEG in rat pups allow for signal recording from only one pup in the litter. Thus, a large number of litters are required to conduct a statistically high-powered study.

The telemetry system currently offers only one channel of differential EEG recording. This limits the possible electrode configurations to differential recording within one brain hemisphere or between the two hemispheres or to one hemisphere with reference to a disparate region such as the cerebellum. This in turn restricts the application of the device to detection of seizures or recording background EEG from one hemisphere. Studies that examine seizure propagation or inter-hemispheric asynchrony cannot be conducted with the current configuration. Another version of the telemetry system that will allow recording of up to 6 channels of data is currently under development. The new configuration will allow experiments that are not possible with the current iteration of the wireless EEG system.

Bandwidth limitations

Our current miniature telemetry system is bandwidth-limited to 0.1 Hz at the low end and to 120 Hz at the high end of frequency range. If the signal frequency falls outside the range of 0.1 to 120 Hz, the signal is attenuated at 12 dB per octave at the high end (>120 Hz) and 6 dB per octave at the low end (<0.1 Hz). This feature of the device limits the amount of noise amplified at the receiver base and enables recordings in relatively electrically noisy environments, such as normal animal facilities. However, due to the bandwidth limitations, the telemetry system is not suitable for studying fast, high-frequency events, such as action potentials, high-frequency oscillations and the electrocardiogram. Instead, the miniature telemetry system is optimized for best

performance while recording EEG in the classically defined EEG bands – 0.1 to 120 Hz. No such bandwidth limitations occur in the wired systems. Effort is being made to increase bandwidth to 4 KHz, albeit with a decrease in transmitter lifetime.

Applications of the telemetry system

Neonatal seizures. The miniature wireless telemetry unit could be used to address many important research applications. In this study, we used the telemetry system to record neonatal seizures in hypoxia and kainate models. Neonatal seizures are a common and serious neurologic condition with poor response to pharmacotherapy. Studies that test new therapeutic approaches for translational drug discovery typically use behavioral seizure scores as the primary outcome measure, without recording EEG (Aujla et al., 2009; Koh et al., 2004; Lai et al., 2009; Mikati et al., 2007; Koh and Jensen, 2001; Folbergrova, 1997, 1994). However, antiseizure compounds often have sedative effects, making behavioral analysis difficult if not meaningless. Additionally, behavioral monitoring can only detect clinical seizures, ignoring electrographic seizures that do not present with a behavior. Electrographic seizures are a serious concern in neonates due to the electro-clinical decoupling that often occurs in this age group (Glass and Wirrell, 2009; Dzhala et al., 2005; Glykys et al., 2009; Boylan et al., 2002; Painter et el., 2009). Rodent models of hypoxia- and kainate-induced neonatal seizures that are monitored with EEG can be used for pre-clinical testing of antiseizure drugs and other therapies given the ability to analyze the

EEG quantitatively. The EEG telemetry system allows detection of both convulsive and electrographic seizures, making determination of drug efficacy more accurate and relevant to the human condition, a key translational component for drug discovery.

Continuous uninterrupted recordings. A unique feature of the telemetry system is the ability to monitor EEG continuously (Figure 2.9). This feature is particularly important in experimental designs that involve, for example, epileptogenesis and progression of epilepsy. Currently, electrographic epilepsy research uses two approaches: *intermittent monitoring*, where recordings are conducted for a number of hours during the day, and *continuous monitoring* where recordings are conducted uninterrupted 24 h per day, 7 days per week. Intermittent recordings require less time to analyze, but they are susceptible to false negatives by “missing” seizures during the periods between recording sessions. This is especially critical when considering that seizures tend to “cluster”. This strategy is suitable for studying the acute period associated with brain injuries, or for experiments that require high numbers of animals with high throughput. Continuously monitored recordings require more analysis yet provide a more comprehensive picture of seizure frequency and duration. Continuously monitoring the EEG is more suitable for tracking disease progression and/or therapy effectiveness, particularly when seizure frequency is low. The miniature telemetry system enables long term monitoring of rodents from neonatal period as they age into adults.

Quantitative approaches. The miniature telemetry system can be used to test seizure detection algorithms and for automated or semi-automated EEG analysis strategies, given the good signal-to-noise ratio of the device and the greatly reduced electrical artifacts. The device also provides excellent recordings of background (or normal) EEG. Clinically, background EEG has been used to predict outcome of various neonatal conditions, including *in utero* asphyxia and hypoxia-ischemia (Selton and Andre, 1997; Murray et al., 2009; Patel and Edwards, 1997; Fitzgerald et al., 2007). Additionally, it can be used to predict the presence of other neurologic insults, such as grading of concussions (Mizrahi and Kellaway, 1984). However, in animal models, the concept of using background EEG as a dependent variable has not been examined. Here we show that by using the quantifiable background EEG patterns, we can track maturation of rat pups from P7 to P11 (Figures 2.7 and 2.8) as a function of age and frequencies in the signal. The age-dependent shift towards higher frequencies as a function of the degree of maturation has been previously reported in human preterm infants (Niemarkt et al., 2011). This proof-of-concept research enables us to conduct future studies of clinically described background EEG abnormalities in animal models of neurologic conditions, such as asphyxia, hypoxia-ischemia, traumatic brain injury and stroke.

Figure 2.1: P7 rat pup implanted with the transmitter of the miniature telemetry device. The transmitter unit was surgically implanted at P6. The transmitter unit is small in size (<1 cc, <1 g), and is designed to fit the top surface area of the skull in the immature rat pup. The form factor of the transmitter is compatible with co-housing of the implanted animals with the dam and littermates.

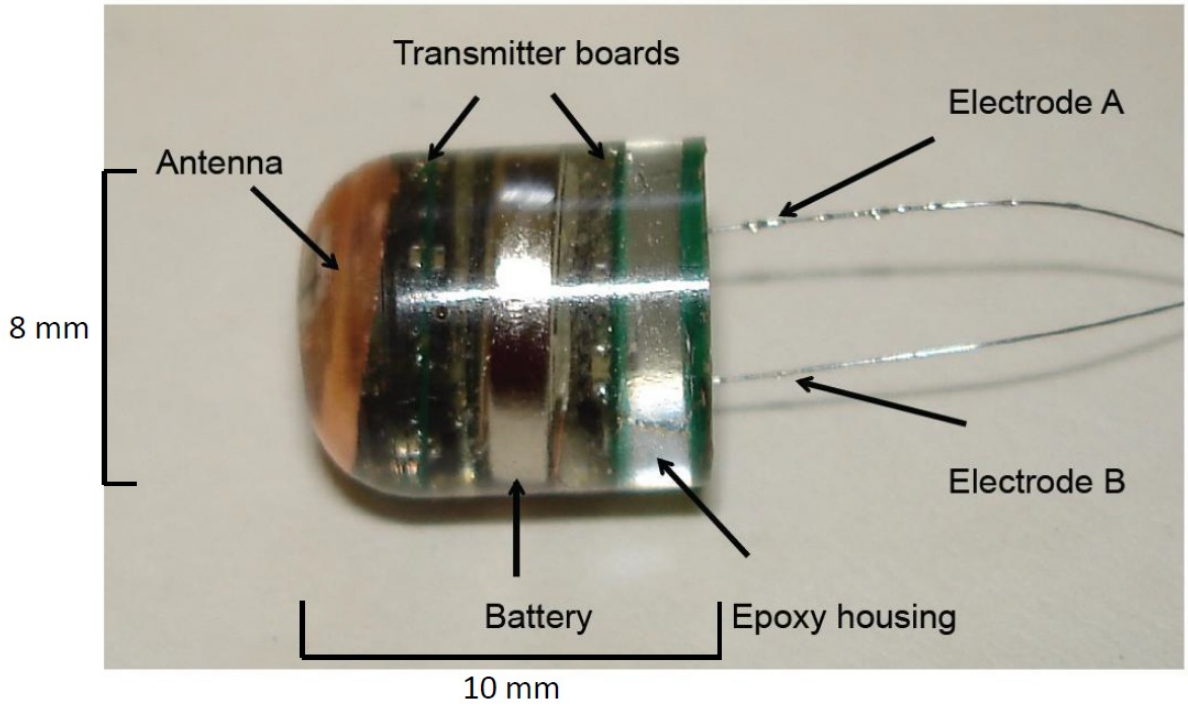


Figure 2.2: Form-factor design of the miniature telemetry transmitter unit. The transmitter consists of two boards with electronic components, a battery, and a flat copper antenna located at the top of the unit. The assembly is encased in a cylindrical epoxy housing. Two electrodes protrude from the housing, with a differential recording configuration (A minus B). The transmitter is implanted on the top of the skull with cut-to-size electrodes located over the surface of the dura.

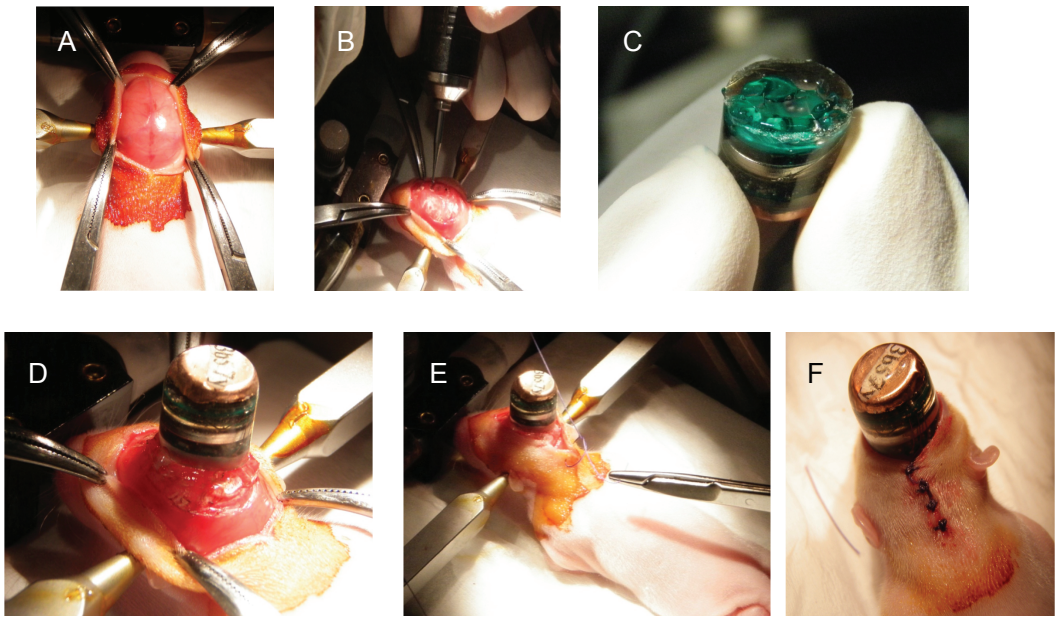


Figure 2.3: Surgical implantation of the telemetry device in a P6 rat pup. The surgery is performed in a sterile field with a stereotaxic frame. An incision is made in the scalp of the animal (A); two burr holes are made with a dental drill (B); the transmitter unit is fixed on the surface of the skull with cyanoacrylate gel and accelerator (C, D); and then, the skin is sutured around the base of the implant (E, F).

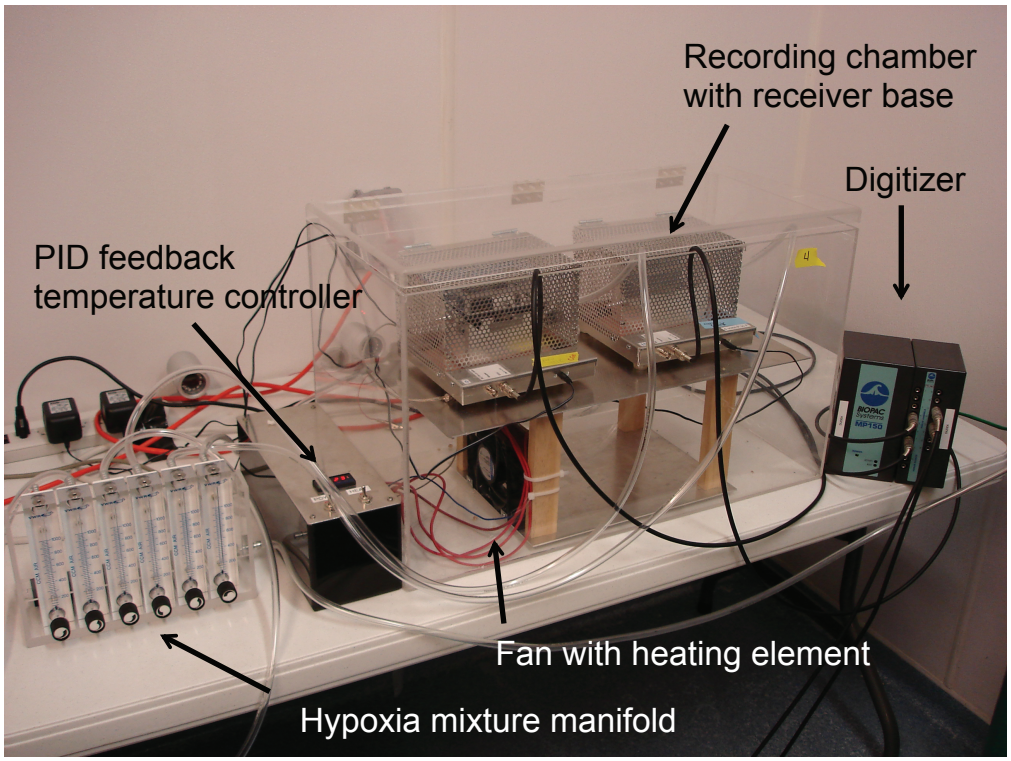
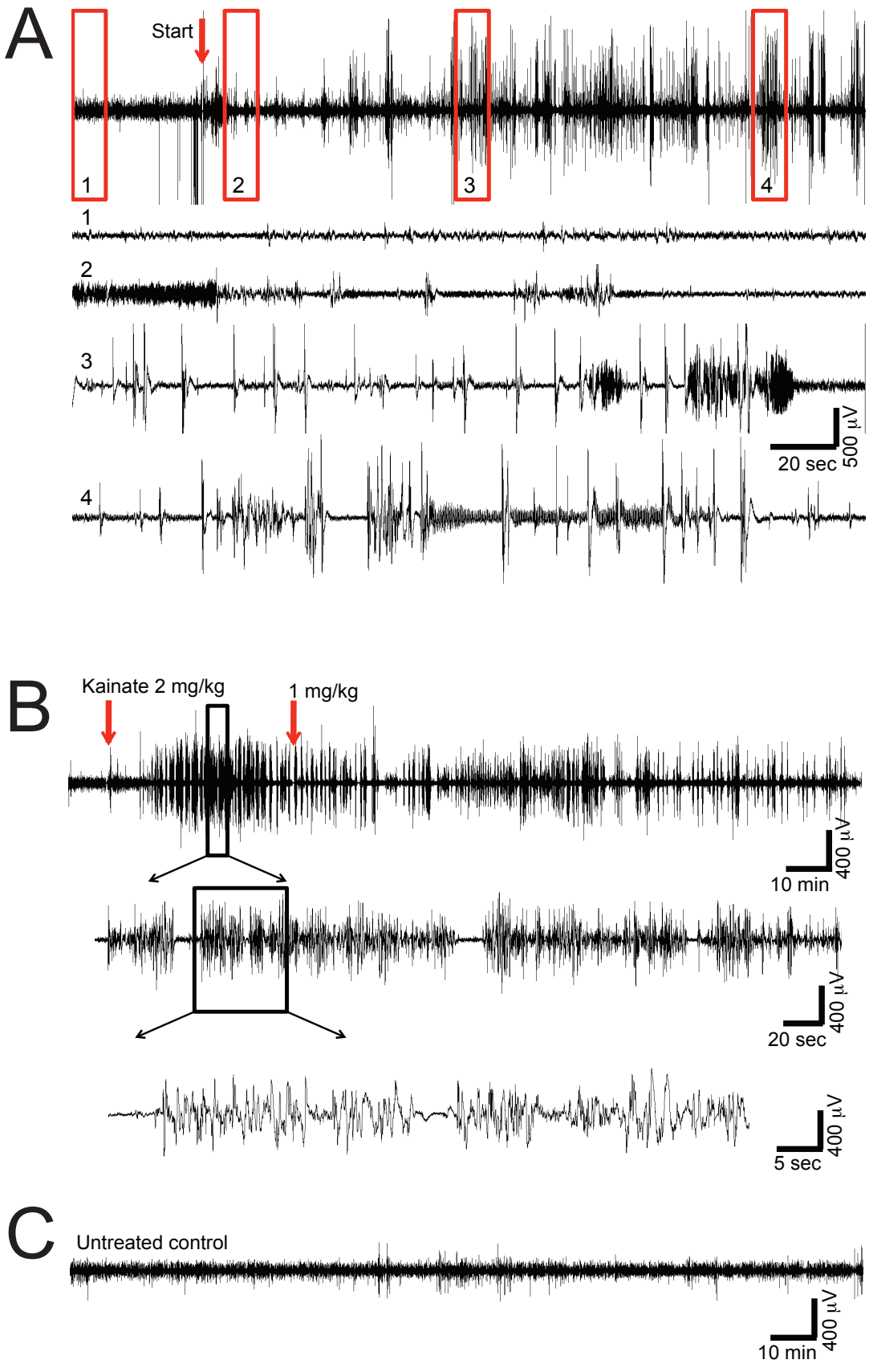


Figure 2.4: Design of the treatment/recording chambers. The chamber is constructed from clear acrylic. A feedback-based PID controller that drives a fan with integrated heating elements regulates the temperature in the chamber. Animals are housed in electrically shielded and sealed recording chambers with a telemetry receiver base. Gases are administered via hypoxia-mixture manifold. Signals from the receiver bases are digitized and recorded with a computer.

Figure 2.5: Typical hypoxia- and kainate-induced seizure activity recorded with the telemetry device. During hypoxia treatment (A), 30 min of baseline was recorded (1) and an 8% oxygen/92% nitrogen gas mixture was administered into the chamber. Hypoxia induced robust electrographic seizure activity throughout the administration of the gas (traces 2-4). In the kainate model, 30 min of baseline was recorded and 2 mg/kg kainic acid was injected IP, with an additional 1 mg/kg after 40 min. Robust seizure activity was recorded after administration of the kainate. Recordings from an untreated control are included for comparison (C).


A
Start
1
2
3
4
1
2
3
4
20 sec
500 µV
B
Kainate 2 mg/kg
1 mg/kg
10 min
400 µV
20 sec
400 µV
5 sec
400 µV
C
Untreated control
10 min
400 µV

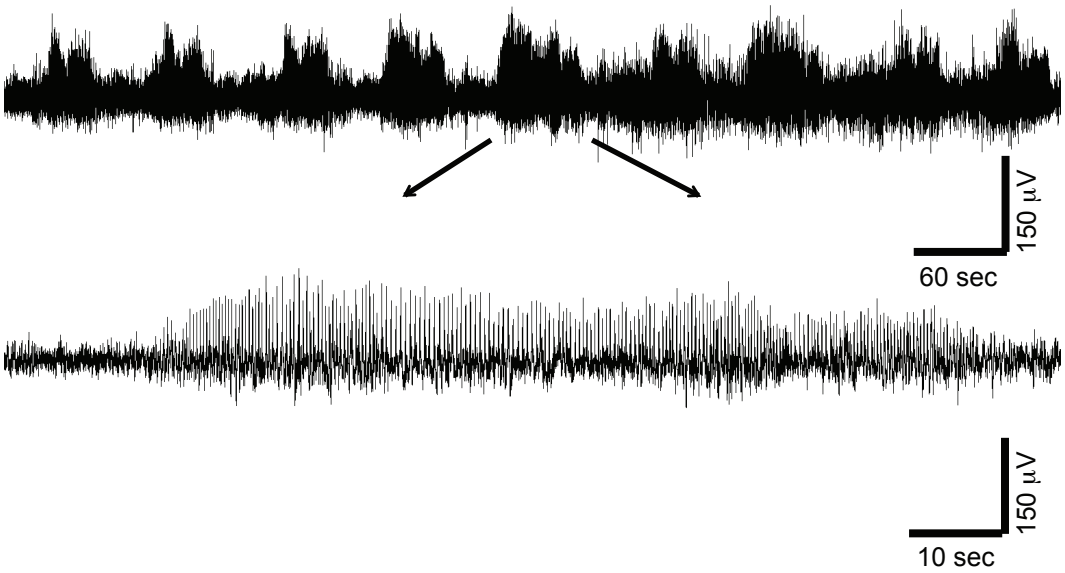
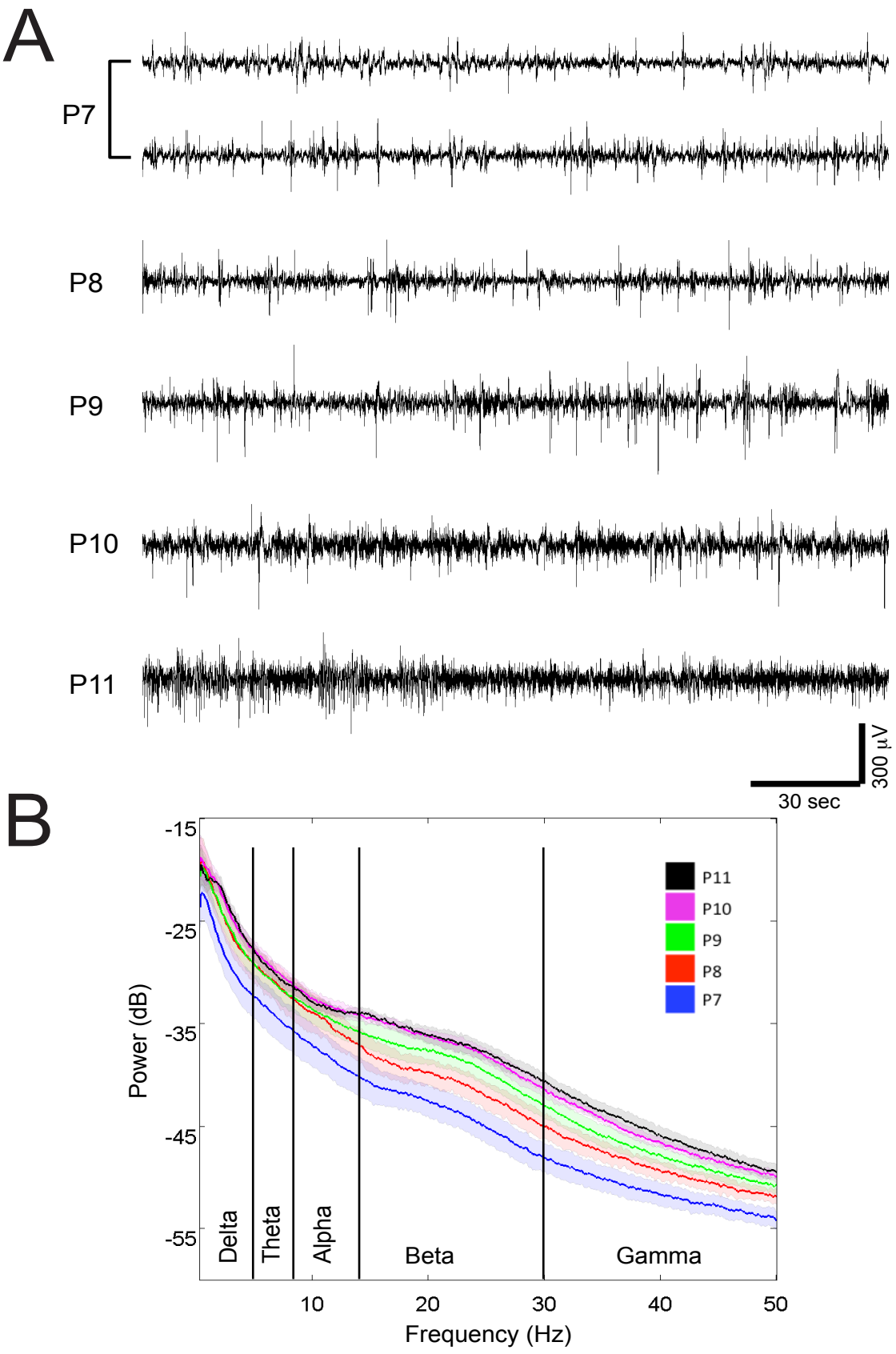


Figure 2.6: Kainate induces seizure activity in a P15 rat pup recorded without burr holes. Animal was implanted at P6 without using electrode burr holes. The electrode wires were placed on the surface of the skull. The animal was treated with 2 mg/kg kainate at P15. Clearly identifiable seizure activity was recorded successfully with this electrode configuration.

Figure 2.7: Age-dependent changes of the background EEG frequencies. Power in the EEG bands was quantified using Fast Fourier Transforms (FFT) and mean values were plotted with 95% confidence intervals across animals. As the animals mature, the power profile of the background EEG changes. A marked increase in power is present from P7 to P8 in all of the frequency bands. The power in the beta and gamma bands increases as the animals mature, with a plateau between P10 and P11.


A
P7
P8
P9
P10
P11
300 µV
30 sec
B
-15
-25
-35
-45
-55
Power (dB)
Delta
Theta
Alpha
Beta
Gamma
10
20
30
40
50
Frequency (Hz)
P11
P10
P9
P8
P7

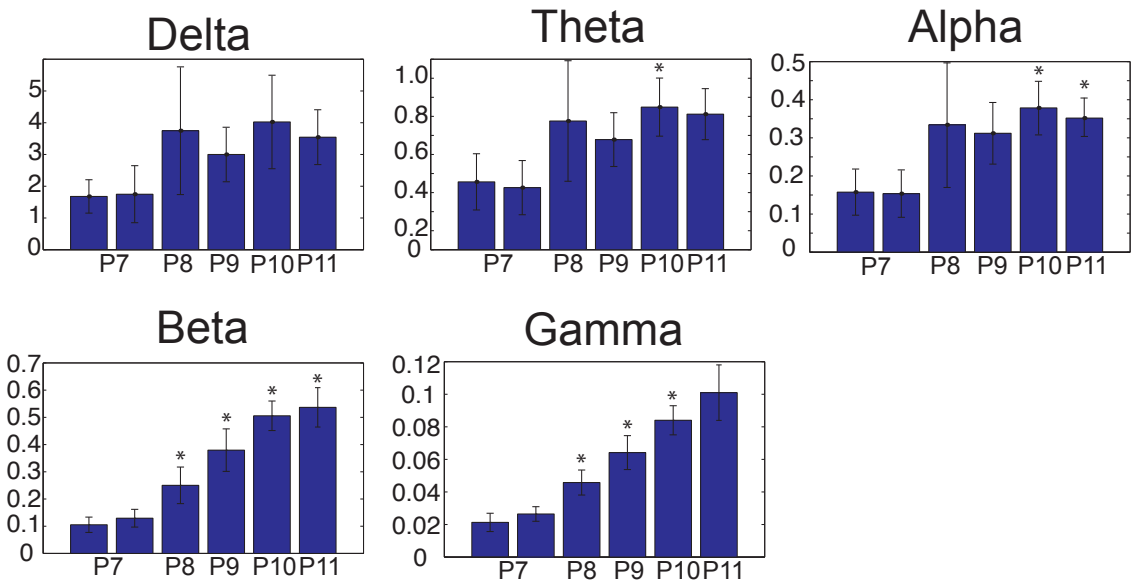


Figure 2.8: Age-dependent changes in the integrated power of background EEG. EEG power was quantified separately in each band by integrating the power under the FFT curve. The age-dependent changes are apparent during animal maturation. Two recordings were conducted at P7 to verify stability and same-day variability of the signal.

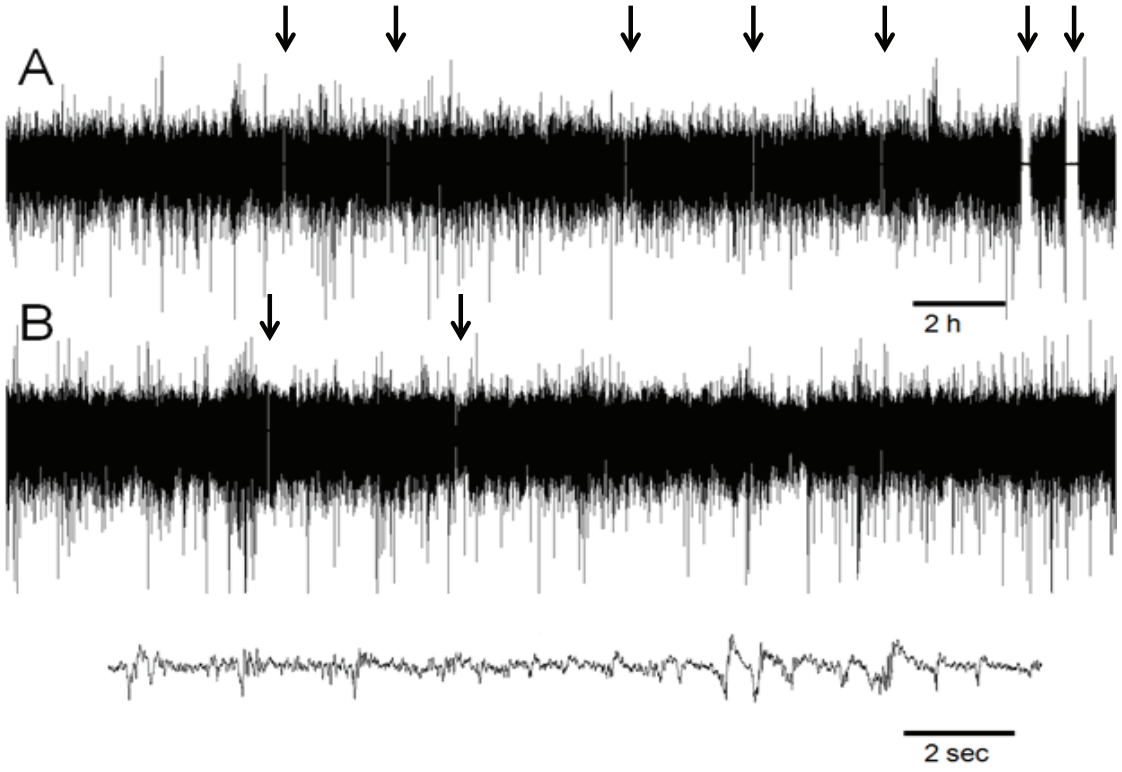


Figure 2.9: Continuous 48-h monitoring in a P7-P8 rat pup. A rat pup was implanted with the telemetry unit at P6 and housed with the dam and littermates in a receiver base designed for an adult animal. A continuous recording of 48 h was made from the animal. Arrows indicate telemetry signal “drop-outs.” This proof-of-concept experiment shows the possibility of conducting continuous uninterrupted recordings in the immature rats.

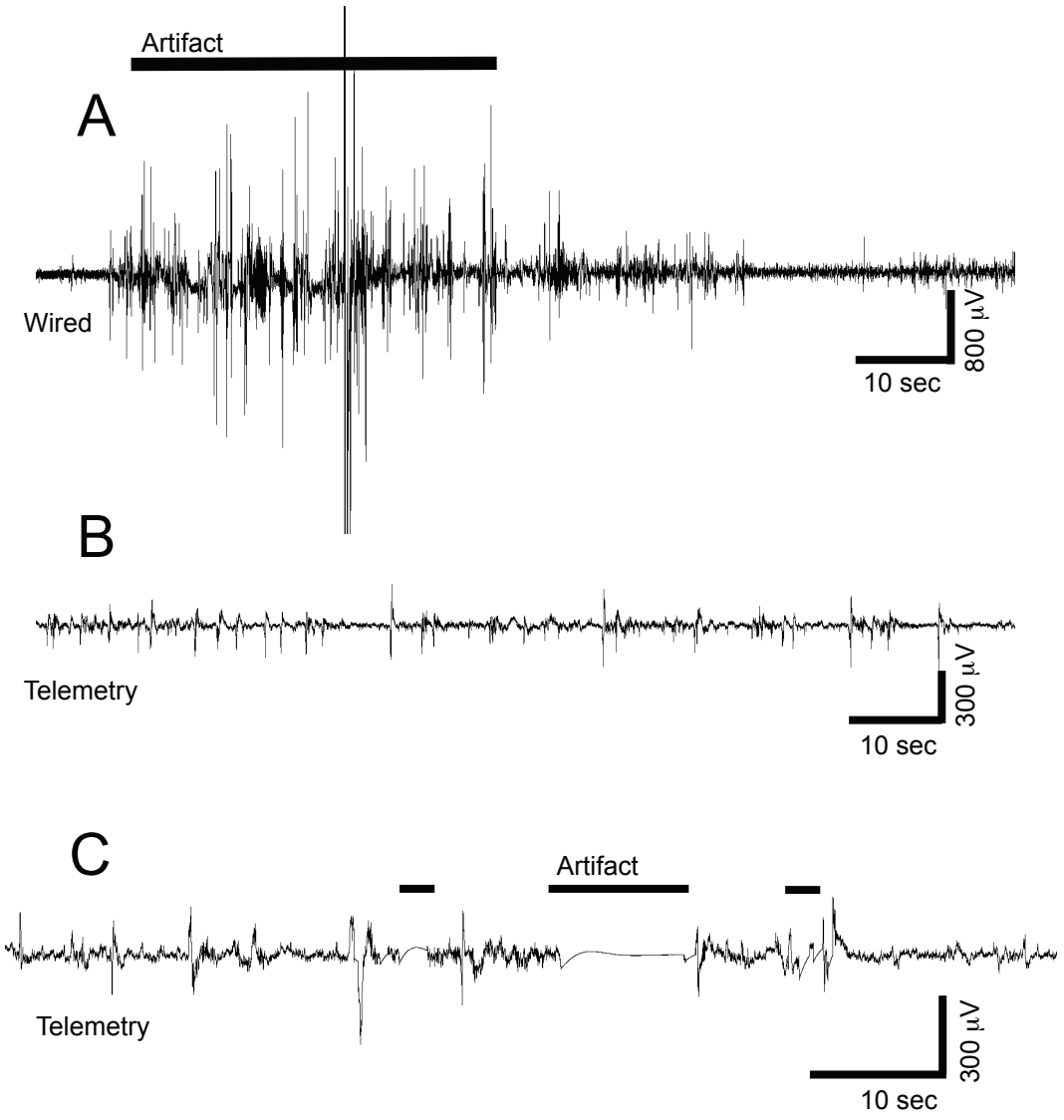


Figure 10: Typical artifacts in a wired and telemetry EEG recording. The wired recording is extremely susceptible to movement artifacts that cause the tether to shift, applying torque forces to the skull of the animal. Because the skull is extremely thin and flexible, these movements appear as large-amplitude artifacts in the signal (A), making analysis difficult if not impossible. Telemetric recording solves this problem by deleting the tether, resulting in a much cleaner signal (B). “Dropout” artifacts (C) occasionally occur in the telemetry recording when the transmitter does not properly couple with the receiver.

Table 2.1: Age-dependent changes in the integrated power of background EEG. EEG power was quantified separately in each band by integrating the power under the FFT curve. Mean power values across multiple animals with standard deviations.

Age	Delta Power	Theta Power	Alpha Power	Beta Power	Gamma Power
P7	1.75±0.9	0.326±0.142	0.154±0.061	0.129±0.033	0.026±0.004
P8	3.75±2.00	0.676±0.316	0.334±0.163	0.250±0.067	0.046±0.008
P9	3.00±0.84	0.578±0.141	0.312±0.080	0.380±0.078	0.064 ±0.010
P10	4.02±1.50	0.749±0.152	0.379±0.070	0.506±0.054	0.084±0.009
P11	3.54±0.86	0.712±0.134	0.352±0.051	0.537±0.072	0.101±0.017

CHAPTER 3

ABNORMAL EEG PATTERNS AND ACUTE SEIZURES RECORDED WITH A MINIATURE TELEMETRY DEVICE DURING ACUTE HYPOXIA AND HYPOXIA-ISCHEMIA IN NEONATAL RATS

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Abstract

In rodent models of hypoxic-ischemic (HI) encephalopathy, the relationship between acute seizures and the resulting lesion is unclear. In the present study, animal models of hypoxia alone (Ha) and HI - both with neonatal seizures - were compared to test hypotheses about neonatal seizures, background EEG and brain damage. In these models, HI results in acute seizures, neuronal degeneration and subsequent epilepsy; however, Ha only causes acute seizures, with no obvious neuronal degeneration or subsequent epilepsy. Sprague-Dawley rat pups were implanted at postnatal day 6 (P6) with a novel miniature telemetry device, and treated for 2 h with HI or Ha at P7. EEG was recorded during the treatment and analyzed using Fast Fourier Transforms. Seizure activity with behavioral correlates was identified as discrete events with high power in delta and alpha EEG bands. In the temporal domain, Ha progressively increased EEG amplitude during treatment, while HI-treated pups showed a progressive decrease in amplitude in delta and alpha bands with significant suppression of background EEG activity. In the frequency domain, HI animals showed attenuation of alpha EEG power during the second hour of treatment, while Ha-treated animals showed a shift toward higher frequencies observed in the alpha band. Thus, although the waveform properties of acute seizure activity were quite similar in both HI and Ha models, Ha-treated rat pups showed a more severe electrographic seizure profile while a progressive attenuation of seizure activity and suppression of background EEG occurred in HI animals. These data support the hypothesis that acute electrographic seizure

activity does not identify neonatal brain damage (and subsequent development of epilepsy); instead, a progressive decrease in background EEG activity over time provides an electrographic sign of neuronal degeneration during an HI insult.

Introduction

Seizures are relatively common in the neonatal period; they are a potential harbinger of profoundly negative neurological outcome, including intractable epilepsy, cerebral palsy, and cognitive deficits. Potential brain insults involving hypoxia alone (Ha) or hypoxia-ischemia (HI) (e.g., perinatal stroke) are thought to be a major cause of neonatal seizures, but metabolic disturbances, encephalitis, and genetic abnormalities can also lead to seizure activity in the neonatal period (Nelson and Lynch, 2004; Mercuri et al., 1999; Sarnat and Sarnat, 1976; Marin-Padilla, 2000; Nelson, 2008, Finer et al., 1981; Malm, 2009; Corey et al., 1988; Sorge and Sorge, 2010). Although considerable data are available on clinical outcomes after neonatal seizures, their predictive value towards determining subsequent neurological outcome is poor, because outcomes can be either benign or devastating. In humans, it is extremely difficult to establish a definitive causal link between the characteristics of the neonatal EEG and the subsequent neurological outcome. Thus, it is unclear whether acute neonatal seizures are a cause of a negative outcome or are merely a symptom of other processes that lead to a negative outcome; and thus, it is difficult to gauge the necessity and efficacy of early clinical interventions to suppress seizures during the critical sub-acute period after neonatal insults (Korotchikova et al., 2011; Murray et al., 2009;

Walsh et al., 2011; Williams et al., 1992; Monod et al., 1972; Scher and Beggarly, 1989; Tharp et al., 1981; Holmes and Lombroso, 1993). Furthermore, nonictal events, such as background abnormalities in the EEG, have been proposed to be useful predictors of outcome, but well designed prospective studies on these features of the neonatal EEG are limited (Holmes and Lombroso 1993, Monod et al., 1972; Tharp et al., 1981). In rodent animal models of Ha and/or HI, the EEG has not been quantitatively classified. Thus, although it is unclear which features of the EEG are associated with frank brain damage and subsequent poor outcome, the presence of prolonged repetitive seizures and/or background abnormalities are the two main types of hypothetical predictors of a negative outcome in both clinical and animal model literature. In this study, Ha and HI in rat pups were used as two complimentary animal models of neonatal seizures. These models are known to have similar behavioral seizures, but virtually nothing is known about the quantitative electrographic properties of neonatal seizures during this acute period. Previous observations indicate that Ha caused little or no obvious neuronal death (Jensen et al., 1992; Rice, 1981) while HI led to an overt hemispheric lesion (Rice et al, 1981). This potentially allows an assessment of the properties of the EEG that may predict neuronal degeneration and frank brain damage. In the present study, single channel EEG was recorded using a novel miniature telemetry system. We hypothesized that both animal models (i.e., Ha and HI) would show robust electrographic seizures during treatment, but HI-treated rat pups will have distinctly different EEG features compared to those found in Ha-treated animals. Previous studies conducted in our laboratory

showed that the presence of a visible infarct is necessary for development of epilepsy in Sprague-Dawley rats subjected to HI (Kadam et al., 2010). The ability to record and characterize the EEG events present in these models may help identify features that are consistent with early epileptogenesis.

Methods

Telemetry unit implantation

Pregnant Sparague-Dawley rat dams were obtained from Charles-River (Wilmington, MA). Rat pups were born in the University of Utah Animal facility. Pups were housed and reared with the dam and implanted at 6-7 days of age (P6-7) with a miniature wireless telemetry system (Figure 3.1). During this procedure, animals were anesthetized with 4% isoflurane, and a maintenance dose of 2% (MWI, Meridian, ID). The stereotaxic unit was sprayed with 70% alcohol, and surgical tools were sterilized by autoclaving and maintained in 70% ethanol. The rat pup was placed in the stereotaxic unit using small-animal ear bars (David Kopf Instruments). An incision was made on the top of the head using a scalpel, and the skin was clamped with hemostats. Periosteum was removed from the skull, and surface bleeding was cauterized. Two holes for electrodes were made using a surgical drill with 0.7 mm burr (Fine Science Tools), 2 mm lateral from midline of the skull, 2 mm apart. The electrode wires of the transmitter system were trimmed to appropriate length and fed through the craniotomies with a target depth at the level of the dura. The transmitter was attached to the skull using a cyanoacrylate gel compound (Loctite 454) with

accelerator (Loctite 7452). Additional cyanoacrylate was applied around the unit and the exposed areas of the skull to stabilize the implant. The skin was then sutured around the implant with Vicryl 4-0 coated polyglactin 910 suture (Ethicon). Animals were injected with 0.5 ml of lactated ringers (subcutaneous), treated with local anesthetic (Marcaine) and were allowed to recover for 24 h with the dam.

Neonatal hypoxia-ischemia and hypoxia treatments

The HI procedure included both male and female Sprague-Dawley rat pups 7-8 days of age (P7-8) previously implanted with the telemetry units at P6. Animals were anesthetized with 2% isoflourane. The ventral midline of the neck was locally anesthetized with marcaine (0.5%, 0.2 mL). A 2-cm incision was made in the animal's neck and the right common carotid artery was exposed by blunt dissection, clamped with micro-aneurism clamps, and cut by cauterization. Clamps were removed after cauterization of the artery. The skin was sutured and the rat pups were allowed to recover in a cage with the dam for 2 h. After a 2-h recovery, the pups were placed in the hypoxia chamber with feedback-controlled temperature control (37°C), and recording chambers (designed and built in-house). Each recording chamber was designed to have an independent gas input and output and a separate antenna for recording. The chambers were then filled with 8% oxygen and 92% nitrogen gas mixture at positive pressure using a pressure-control manifold. Temperature was verified independently from feedback control using a Vernier Instruments thermocouple. The rats were

exposed to this mixture for 2 h, and were then allowed to recover with the dam and littermates. Ha-treated pups had a sham neck incision and were subjected to 2 h of hypoxia (8% oxygen and 92% nitrogen) with no ischemia. During the treatment, EEG was continuously recorded (Figure 3.2). After treatment, the pups were given 0.5 ml lactated ringers. Animals were returned to the dam and euthanized 72 h after HI to verify the presence of the lesion.

Data acquisition and recording

The wireless device amplified differential signals from two integrated wire electrodes with a bandwidth of 0.1 – 120 Hz and transmitted the EEG signal to the receiver bases through capacitive coupling. The signals from multiple receivers (one per animal) were then digitized by a Biopac MP150 (Goleta, CA) analog-to-digital converter, sampled at 500 Hz, and stored on a PC computer using Acknowledge 4.1.1 (Biopac) software.

Signal analysis

EEG recordings from rat pups during treatment were analyzed in both the temporal and frequency domains. First, events were manually separated and classified into categories based on high power in EEG frequency bands using Acknowledge 4.1.1 software (Biopac, Goleta, CA). Three categories were established – abnormal delta, abnormal alpha and background. The data files were then converted into MATLAB format (Mathworks, Natick, MA). RMS power was calculated for each event in these categories and was plotted as a function

of time. The RMS value is the square root of the arithmetic mean of the squares of the EEG amplitudes over time. The data were then binned into 10-min bins for seizures and 5-min bins for background, and the means of each bin were plotted over time. Statistical differences were determined between HI with Ha events over the full 2-h treatment and during each hour of the treatment. A one-sample Kolmogorov–Smirnov test (K-S, P>0.05 to reject) was used to test the null hypothesis that the distribution of RMS power for each comparison could be drawn from a normal distribution. In instances in which data passed the K-S test (i.e., assumption of normality), independent-sample Student's t-tests were used to test the differences in mean power (P>0.05 to reject). In instances where the distribution of RMS power did not pass the K-S test, a Mann-Whitney U test was used (P>0.05 to reject). Fast Fourier Transforms (FFTs) were performed to analyze EEG data in the frequency domain from 0 to 20 Hz. Power spectral densities (PSDs) were estimated from the FFT using 256 Hann-window segments based on the Welch method (NeuroExplorer, Littleton, MA) and normalized by $10*\log_{10}$ (PSD). Power levels at all frequencies in 0.1 to 20 Hz were plotted with 95% confidence intervals.

Results

Abnormal seizure-like behavior during HI/Ha treatment

Animals that were treated with HI (n=12) and Ha (n=9) presented with similar behaviors during treatment. Ha-induced convulsive behaviors started within minutes of hypoxia-mixture administration. Both groups exhibited abnormal

behaviors that could be classified into three categories: 1) convulsions 2) “shiver-like” behavior, 3) complete behavioral arrest. The behaviors were similar in both groups (i.e. both HI and Ha were abnormal). Because the pups were immature and lacked motor development, these seizures could not be classified using standard Racine scale (Racine, 1972). Untreated EEG-implanted animals did not exhibit any of the above-described behaviors. Behaviors in untreated control animals included body flexion, extension and regular myoclonic jerks. These were identical in both EEG-implanted and un-implanted, untreated animals. No convulsive activity was detected in animals that were not exposed to hypoxia. Following the administration of HI/Ha, the animals recovered within hours and behaved normally. Two animals died during administration of HI; nonetheless, their seizure activity was analyzed. Because the behavioral profile of each group was largely similar, this study focused on EEG recorded from the treatment groups.

HI and Ha induce qualitatively similar electrographic seizure activity

To determine whether treated animals had specific electrographic abnormalities, we recorded and qualitatively analyzed EEG during the administration of the HI and Ha. During the course of the treatment with HI and Ha, the EEG patterns were similar to each other but dramatically different from the activity that has been observed in control animals (Figure 3.3). Three distinct EEG patterns were apparent in the signal. First, was the high amplitude delta frequency band (0.1 – 4 Hz) discharge, which occurred during classic convulsive-

like behavior (Figure 3.4A). These discharges were 30 sec to minutes long in duration. The second type of discharge that could be observed during treatment was an event with high power in the alpha frequency band (8-13 Hz, Figure 3.4). This type of event was accompanied by “shivering-like” behavior. The seizure activity occurred both as discrete events (Figure 3.4A) at the same frequency band (i.e. delta or alpha) or as combinations (Figure 3.4B) of both types of discharges in an alternating pattern. The events fit in the categories described and could be differentiated using band-pass filtering (Figure 3.4C). The third type of discharge was a low amplitude activity with power in 2-4 Hz frequency band (Figure 3.5). This last pattern lasted for minutes and had no behavioral correlate. The three types of events were present in HI and Ha treated groups but were absent in the untreated control animals. The qualitative presence of each of these types of events could not be used as a biomarker for presence of brain damage, as these events were universally present in each of the treatment groups.

HI and Ha treatment have different quantitative temporal profiles

To further investigate the differences in electrographic profiles of HI- and Ha-treated animals, we examined the RMS power properties of each abnormal event as a function of time during treatment. To perform this analysis, EEG traces were visually separated into discrete abnormal events and portions of background activity between these events (Figure 3.6). The abnormal discharges were classified into events with dominant power in the delta (0.1-4 Hz) and alpha

(8-13 Hz) frequency bands. The RMS power was then calculated for each of these events for all animals. RMS data from all animals were then binned in 10-min intervals for alpha and delta bands and 5-min bins for background EEG over the entire 2-h period of treatment. The average number of events in the HI group during the first hour was 24±9.6, and 15±4.9 during the second hour (Figure 3.7); the difference was statistically significant (p=0.02). In the Ha group, the average number of events across animals was 28.9±5.2 during the first hour and 35.6±13.8 during the second hour; the difference was not statistically significant (p=0.12). Across groups, the difference between the first hour of HI and the first hour of Ha was not significant (p=0.16); however, the second hour of HI was significantly different from the second hour of Ha (p=0.004) (Figure 3.7). RMS power was dominated by high-power delta events (Figure 3.8A, 3.8C). RMS power of these events was not statistically different during the first hour of treatment (p=0.904); however, during the second hour, differences emerged (p= 0.006). During the second hour, the RMS power of the delta events in HI animals dropped below that of the Ha-treated rats, while in Ha animals the power profile steadily increased over time and was significantly different from the first hour (p=0.001). These data fit with the previously described timeline of the progression of brain damage in the HI model, in which brain damage was reported to be histologically confirmed to begin 90 min after start of HI treatment (Vannucci et al., 1990). The pattern of the temporal distribution of alpha events was different from that of delta. The differences between HI and Ha treatment became apparent 20-30 min into the treatment (Figure 3.8D), however mean

RMS power was statistically different only during the second hour (p=0.002), when the two profiles were compared. The RMS power profile of background EEG in HI animals followed the pattern of alpha events, with differences in RMS power beginning at 20-30 min following the start of treatment (Figure 3.8B). The quantitative decrease in RMS power during this time was consistent with qualitative suppression seen in the EEG traces. In Ha animals, the RMS power of alpha events remained relatively stable over time. These data suggest that the temporal decrease in RMS power in background EEG 30 min after the start of HI is a potential sign of the impending neuronal injury.

EEG power in the frequency domain

To determine EEG features in the frequency domain, the recordings were divided into first and second hours of treatment. During the first hour (Figure 3.9A), the frequency profiles of HI and Ha were nearly identical. The signal had slightly higher power in the delta band, and a power increase in the alpha band compared to control (n=7) (Figure 3.9A). During the second hour of treatment the frequency profile changed. In Ha-treated animals, the overall power in delta and alpha bands further increased, while in HI-treated animals there is a significant drop in power in these EEG bands (Figure 3.9B). Overall, the power in all frequency bands remained higher due to seizure activity in treated animals compared to the control animals. When power spectra were examined within each group between the first hour and second hour, alpha-band attenuation could be seen in HI-treated animals (Figure 3.10A) during the second hour. In

Ha-treated animals, the power increased over all frequency bands during the second hour, with high power alpha band shifting towards higher frequencies (Figure 3.10B).

Discussion

Hypoxia drives acute electrographic neonatal seizures

The use of the miniature telemetry system in the present study has allowed electrographic recording of seizures and EEG background during Ha and HI treatments in neonatal rat pups. In human neonates, the relationship between different types of neurological insults (e.g., hypoxia, ischemia, and other forms of possible brain injury) and the generation of *electrographic* neonatal seizures is unclear. Previous research on neonatal seizures in P7-P12 rat pups has been based primarily on behavioral monitoring and has suggested that Ha - without any obvious brain damage – can induce robust acute seizure activity (Jensen et al., 1991; Jensen et al., 1995). The data presented here indicate that severe electrographic seizures occurred during 2-h exposures to hypoxic environment in both Ha and HI animals. These data strongly suggest that hypoxia is the critical component of the insult that drives the electrographic seizures, and that brain injury - with the progressive occurrence of acute neuronal death - does not appear to be as important a factor for the acute generation of repetitive electrographic seizures. Thus, the present data argue that Ha is a more robust model than HI for generation of electrographic seizures in the neonatal brain. Combined with the use of wireless telemetry, Ha may be a better model of pure

acute seizures, making it an excellent candidate for testing of therapies for the treatment of acute seizures.

Neonatal seizures and brain damage

The observation that Ha induced robust electrographic seizures, even though no obvious neuronal damage occurred, could be interpreted to imply that Ha-induced seizures at P7 did not lead to overt neuronal damage. However, the occurrence of seizures in an already damaged brain, arising from mechanisms such as HI and traumatic brain injury, may make the damage *that has already occurred* worse. Previous work using a HI model - with or without kainate-induced seizures after HI treatment - provided evidence that seizures exacerbate brain damage (Wirrell et al., 2001). Thus, a critical interpretational caveat for the present data set is that electrographic seizures, independent of behavioral convulsions, may worsen the neuronal death associated with the prior brain insult – even if the seizures themselves do not cause overt damage. The present data also suggest that ongoing neuronal damage during seizures serves to *attenuate* the seizures temporally, as observed by reduced seizure frequency and intensity. This reduction in seizures is possibly due to HIE-induced massive depolarization across a network that effectively blunts seizure recurrence.

These data suggest that the generation of seizures is associated with hypoxic excitation of neurons, and that as long as these neurons maintain a large negative resting potential, the hypoxia drives intense seizures. On the other hand, when profound brain damage would occur during HI, the seizures would

actually decline over time, presumably because of a profound depolarization-inactivation of neurons across the large area of the neuronal damage. Here, repolarization rather than further depolarization could be what is driving the seizure activity. This speculation is supported by our finding that in HI animals, while seizures actually relented, the overall EEG pattern exhibited suppression of the background activity (i.e., depolarization-inactivation may have caused background suppression, and rebound excitation may have caused seizures). It seems likely then that the penumbra, the area surrounding the most intensely damaged zone where the injury was not so profound, may have been the region where seizures were particularly active. If these seizures caused further damage, they could lead to poor clinical outcomes. Thus, our data are consistent with the view that seizures can be associated with a poor clinical outcome, but as thought before, seizures *per se* did not appear to directly cause overt brain damage in the present models.

Biomarkers for neonatal brain damage

The observation in the present data that profound electrographic seizures during Ha are not associated with overt brain damage also argues that seizures *per se* are not a good biomarker of brain damage. Furthermore, although the occurrence of kainic acid-induced seizures with HI-induced brain damage (as observed by Wirrell and co-workers; 2001) may exacerbate the amount of damage from the original HI insult, the observation here that seizures declined during the HI insult (when presumably the brain was undergoing damage) further

suggests that seizures are not a good biomarker of acute brain damage in the neonatal brain. Alternatively, we observed that during HI (but not during Ha), the progressive suppression of the seizures during the 2-h treatment period was associated with suppression of the background EEG. Background suppression has frequently been observed in human neonates with an HI infarct, but has been rarely observed in animal models of neonatal HI seizures. Therefore, the present data - made possible through the use of the miniature telemetry system - argue that background suppression of the EEG is a biomarker for ongoing brain damage in the neonatal brain. Thus, an important concept that emerges from the present data is that acute electrographic seizures alone do not herald subsequent brain damage; however, the occurrence of seizures with the background suppression of the EEG is a robust marker of brain damage.

Critical timing in the progression of injury

A major challenge in perinatal stroke management is that quick and sensitive biomarkers for predicting the severity of a potential neurological outcome currently do not exist. However, there is evidence that rapid decisions and timing are critical in deciding upon intervention. Evidence suggests that in fetal sheep, the HI injury is not linear, but rather is a complex multi-stage process with EEG activity suppression preceding epileptiform activity. The most severe injury, such as laminar necrosis, was reported to be detectable after epileptiform events (Williams et al., 1992). We can theorize then, that an intervention during the initial suppression can attenuate brain damage and improve clinical outcome.

Based on our data in the rat model, we see similar suppression of EEG background beginning 20 min after induction of HI. While we did not quantify the timing of the injury ourselves, it has been shown previously that neuronal necrosis begins to occur 90 min into the hypoxia treatment (Vannucci et al., 1990). Additionally, new clinical data suggest that quantitative analysis of background EEG can be useful in predicting the degree of encephalopathy (Korotchikova et al., 2011). Our animal model, combined with high-quality EEG recordings made possible by the miniature telemetry system, suggests that suppression of EEG is a more specific indicator of brain damage compared to the presence of seizures alone.

Quantitative relationships between brain damage and EEG

An important component, if not a limitation, of the present study is the binary nature of the study design. By using Ha versus HI with specific methodology designed to differentiate brain damage and no brain damage, we were able to use Ha without brain damage as a mechanism of evoking profound electrographic seizures. On the other hand, background suppression of the EEG, which obviously cannot be ascertained from behavior, but only by electrographic recording, is a robust marker of brain damage. We can speculate that the timing of the start of background suppression and initial drop-off in alpha power is curious, as it seems to mirror a biochemical reduction of phosphocreatine in affected area of the brain, as reported by Vannucci (1990). Thus, initial background suppression and changes in alpha may be signs for metabolic

changes preceding cellular degeneration, and the decrease in the power of delta EEG events may be a biomarker for the genesis of overt neuronal degeneration. The initial timing of background suppression can potentially be used as an intervention point for testing of therapies that would improve outcome. What is unclear at this point is the spatial relationship between seizures and background suppression, and the degree to which lesions of various sizes reflect different degrees of EEG abnormalities. Further work with more recording sites and better control of lesion size - as an independent variable - should shed light on the issue of the relationship between seizure activity, background suppression, and brain damage. The binary nature of our study let us establish a detection method for catastrophic injury. Further studies will enable us to refine this concept to be more specific.

Conclusions

Overall we have shown that Ha can powerfully drive neonatal seizures without obvious brain damage, but neonatal seizures can also be seen during HI, when brain damage is occurring. The acute seizures had clearly definable frequency bands of activity, independent of whether brain damage occurred or not. The fact that seizures occur during Ha and HI suggests that the acute seizures alone are not good biomarkers of brain damage. However, the suppression of background EEG, which occurs exclusively during HI, strongly argues that background abnormalities in the EEG are a much better sign of neonatal brain damage in our model. Future studies may be able to more easily

quantify the electrographic activity associated with neonatal seizures, relating them to potential alterations in subsequent cognitive/motor deficits and/or increased seizure susceptibility.

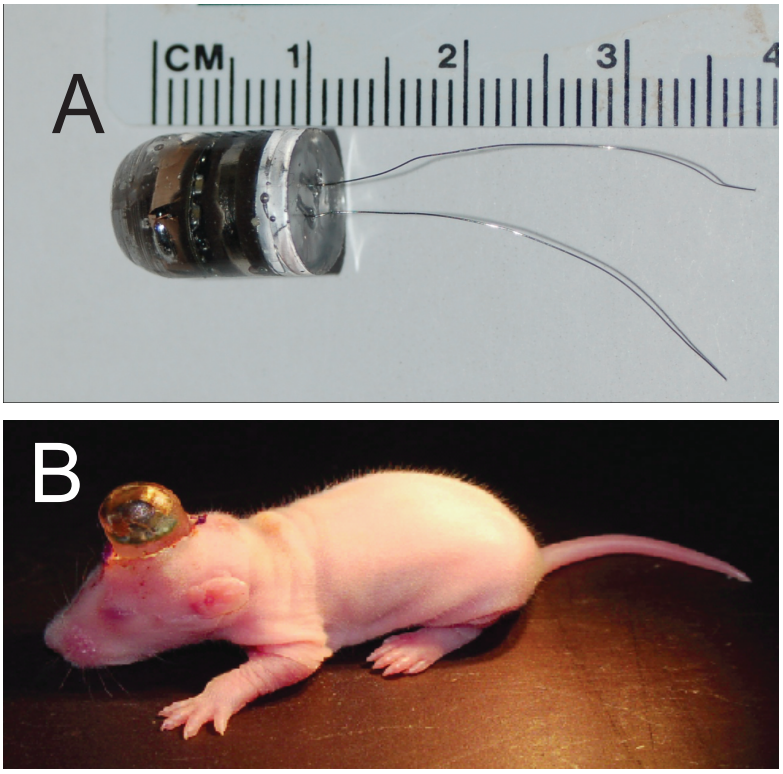

Figure 3.1. Custom-made, miniature telemetry system. The device records EEG activity in freely moving rat pups with minimal impact on the rearing environment of the animals. The small size (<1 cc) and weight (<1 g) (A) of this system allows attachment of the transmitter and electrodes (A) to the skull for recording EEG from rat pups (B) at as young as postnatal day 6 (P6).

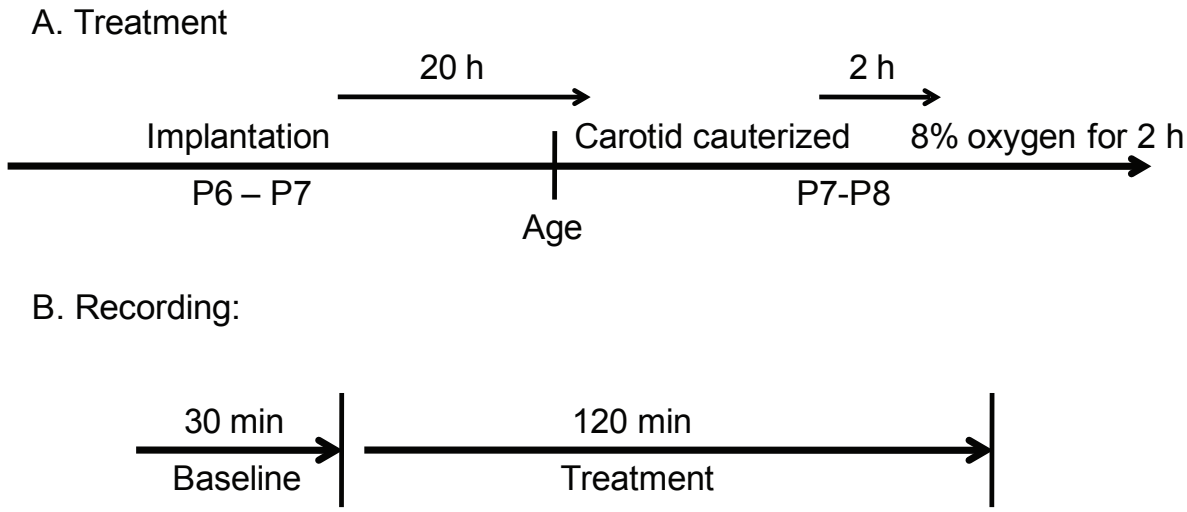

Figure 3.2. HI/Ha treatment and recording protocol. Rat pups were implanted with the device at P6 and were allowed to recover overnight. On the next day, their right common carotid artery was cauterized and the hypoxia-gas mixture was administered after they were allowed 2 h for recovery (A). In animals of the Ha group, similar steps were performed, but the carotid artery was left intact. EEG baseline was monitored for 30 min before administration of Ha and for 2 h during the treatment (B).

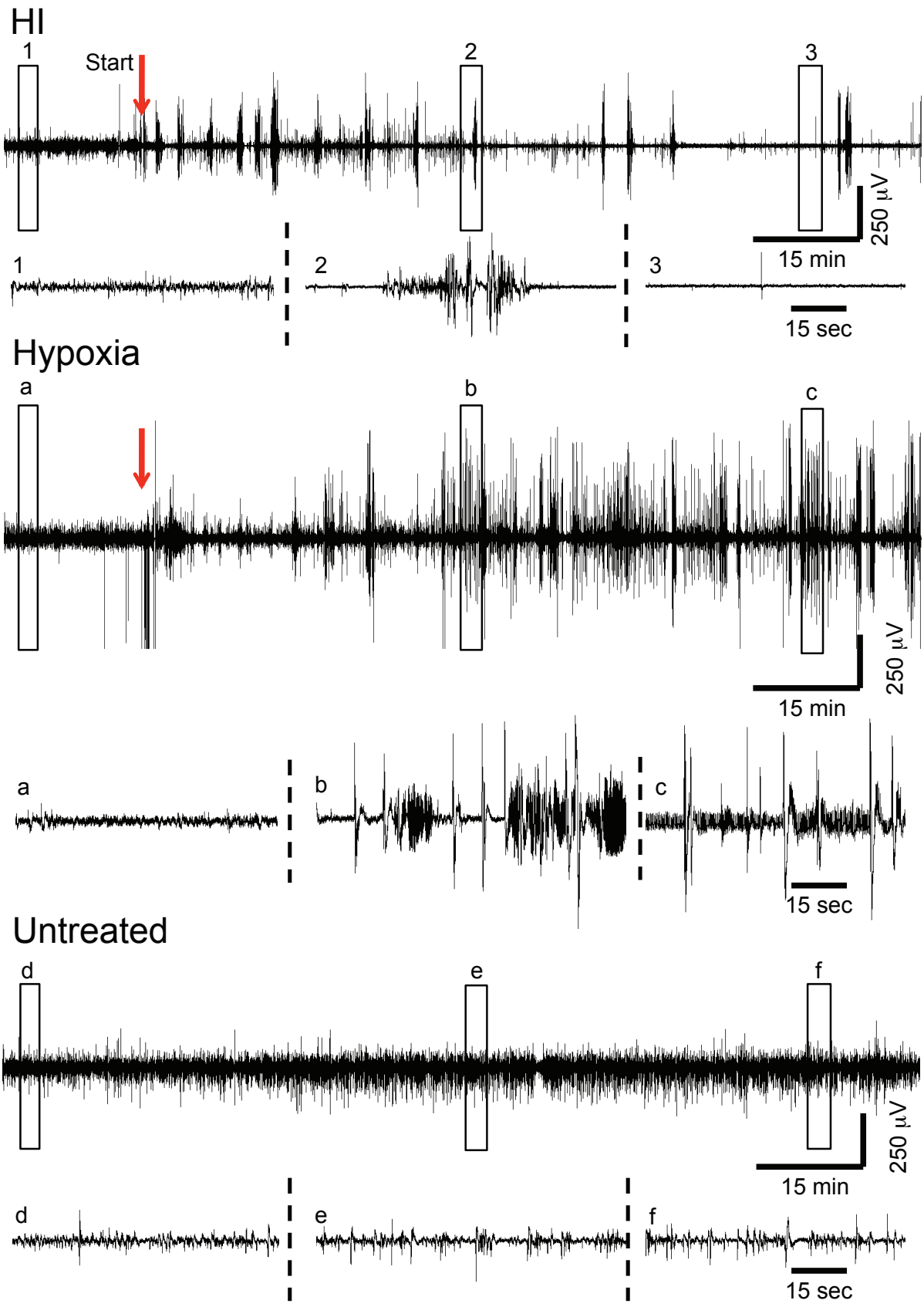
HI
1
Start
2
3
250 µV
15 min
1
2
3
15 sec
Hypoxia
a
b
c
250 µV
15 min
a
b
c
15 sec
Untreated
d
e
f
250 µV
15 min
d
e
f
15 sec

Figure 3.3. EEG activity during the administration of HI and Ha, as well as in a normal animal. During the 2 h of hypoxia administration, HI and Ha animals had distinct and consistent patterns of EEG activity. Prior to administration of hypoxia, all animals had normal EEG patterns (1, A). EEG in HI-treated animals contained high-voltage discharges with suppressed background (2,3). Ha was characterized by high voltage abnormal ictal activity without attenuated background (B, C). Untreated controls had normal EEG background with some typical oscillatory discharges (D, E, F).

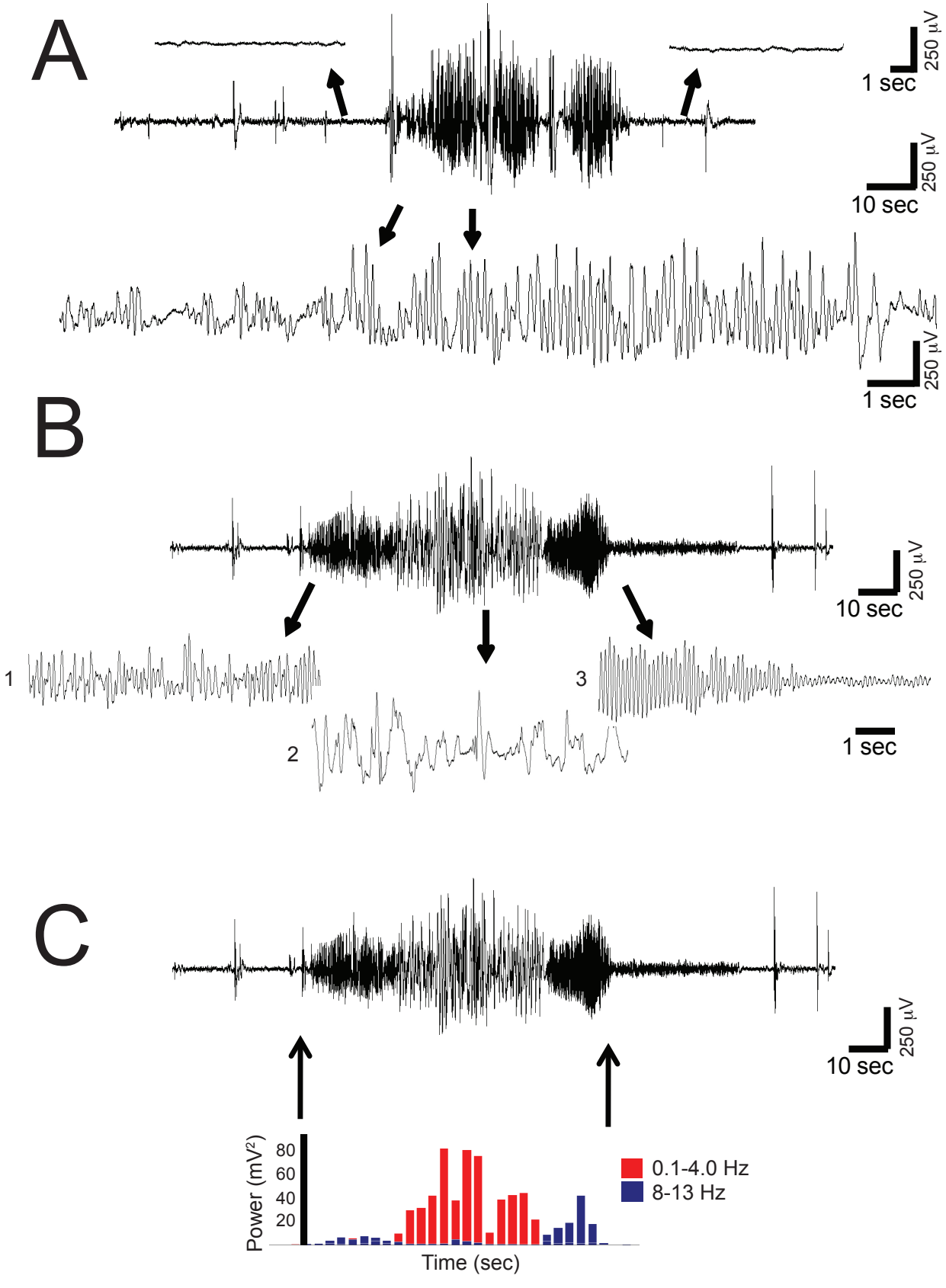
A
250 µV
1 sec
250 µV
10 sec
250 µV
1 sec
B
250 µV
10 sec
1
2
3
1 sec
C
250 µV
10 sec
Power (mV²)
80
60
40
20
0.1-4.0 Hz
8-13 Hz
Time (sec)

Figure 3.4. High-amplitude EEG discharges recorded during Ha and HI treatment in P7 rat pups. The two dominant types of discharges were characterized by their power in the frequency bands. The discharges occurred with most of the power in the alpha (8-13 Hz, A, 1, 3) and delta bands (0.1 to 2 Hz, B2). The activity could be discrete (A) or a combination of events with power in both alpha and delta bands (B). The types of discharges could be discretely separated and classified by band-pass filtering (C).

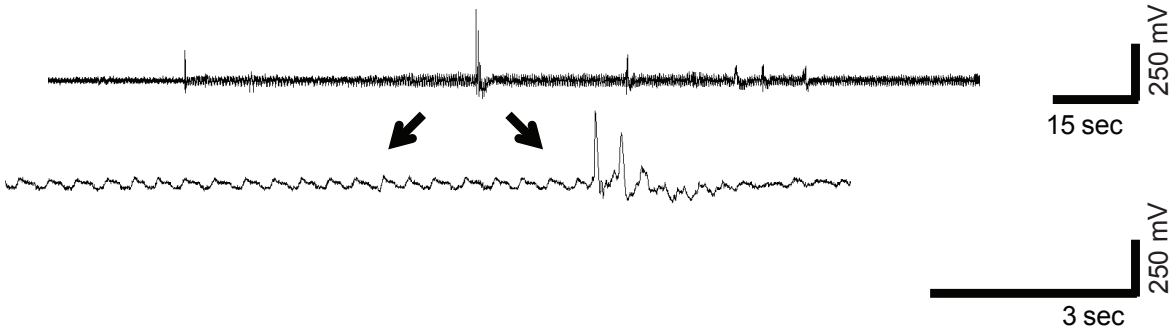

Figure 3.5. Low-amplitude discharges during Ha/HI treatment. The low-amplitude events had peak frequency in the delta frequency band and primarily occurred during the 2nd hour of the treatment.

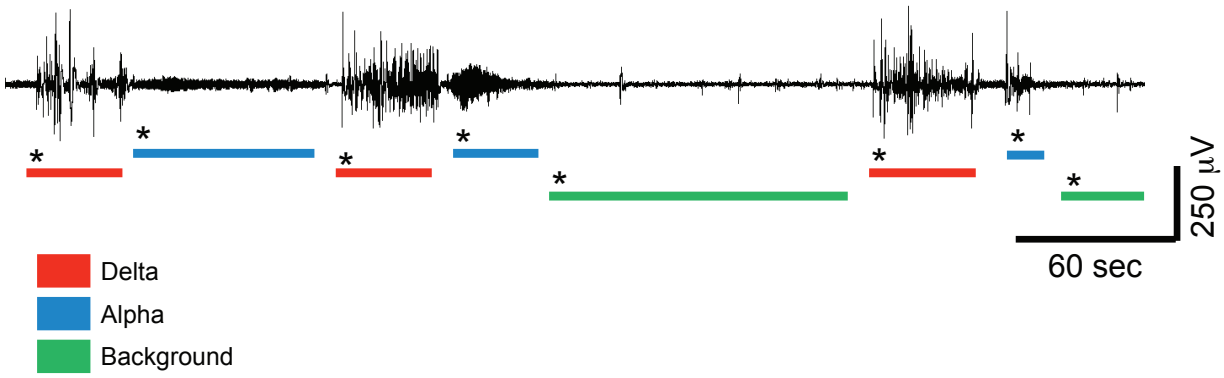

Figure 3.6. Separation and analysis of the EEG events. All EEG traces were manually separated and classified into seizures and background. Seizures were divided into categories based on their frequency band (Delta, Alpha). Start time of each seizure or inter-seizure background was used as a basis of analysis in the temporal domain.

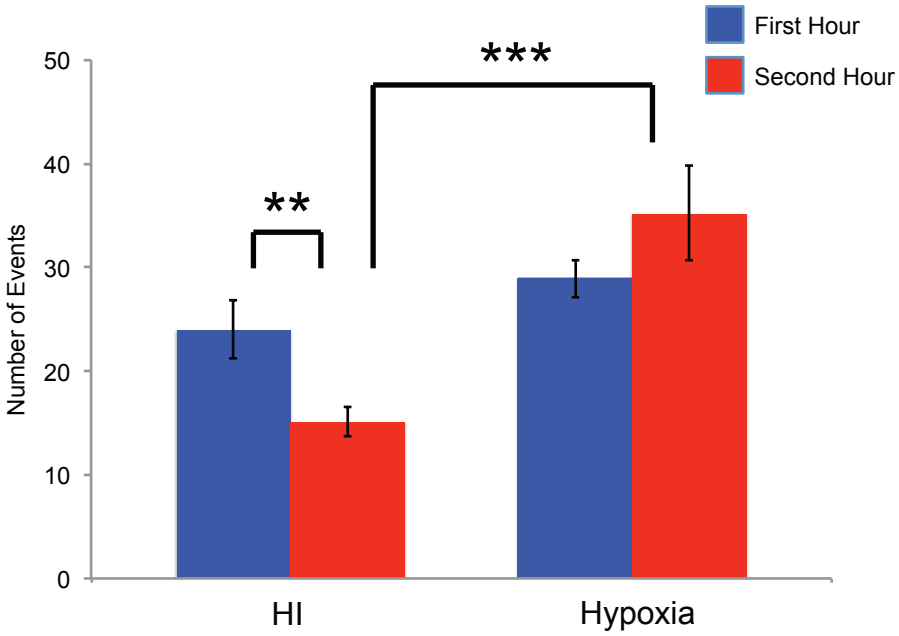

Figure 3.7. Total number of abnormal events during first and second hours of administration of HI and Ha. Number of abnormal events in delta and alpha frequency bands was counted and the averages during first and second hours were plotted and compared using t-test. ** corresponds to $p < 0.05$, *** to $p < 0.005$

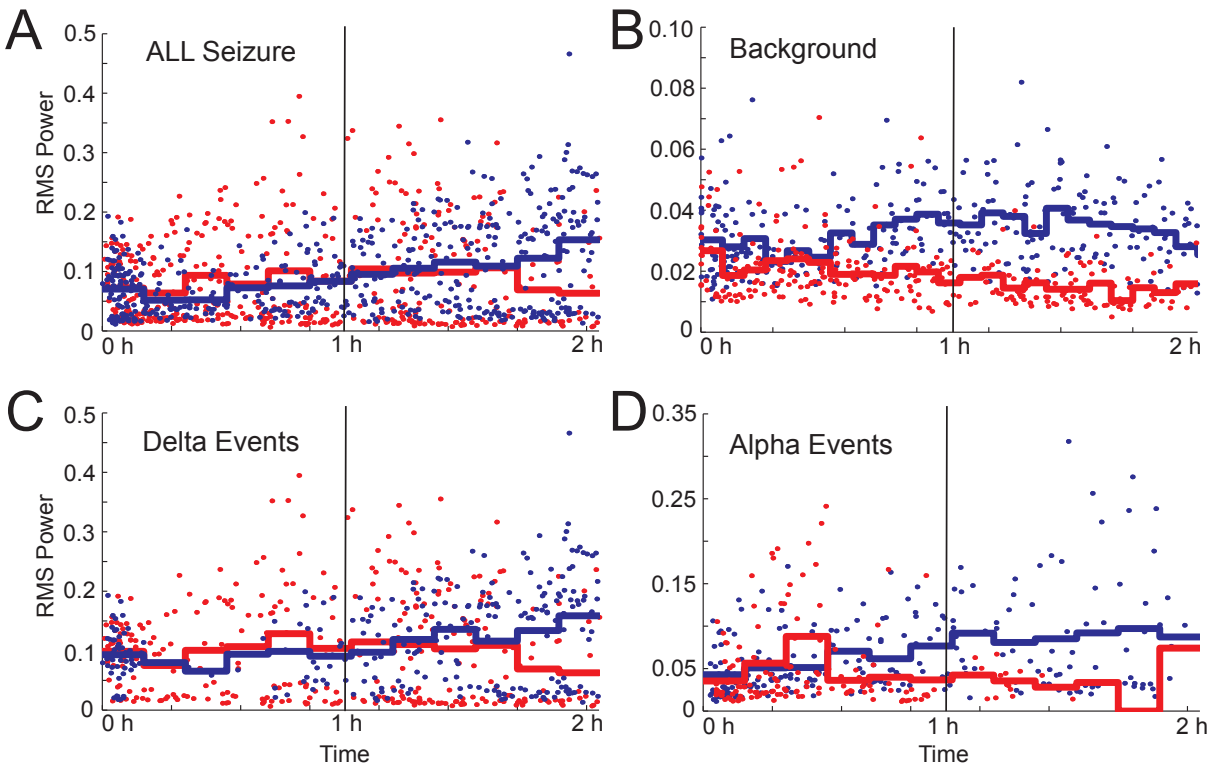

Figure 3.8. Temporal distribution of event RMS power during treatment. Each seizure and background portion was plotted based on the time of the start of each event and its RMS power. The time was divided into 10-min bins for events and 5-min bins for the background. The means of each bin were plotted as a line on the graph. In all seizure events, the temporal profiles were similar until about 90 min into the treatment (A). This profile appeared to be dominated by events in the delta frequency band (C). Alpha events ($p=0.002$) and background ($p<0.001$) profiles were showing differences as early as 20 min after the start of the treatment (B, D). See table 1 for p values.

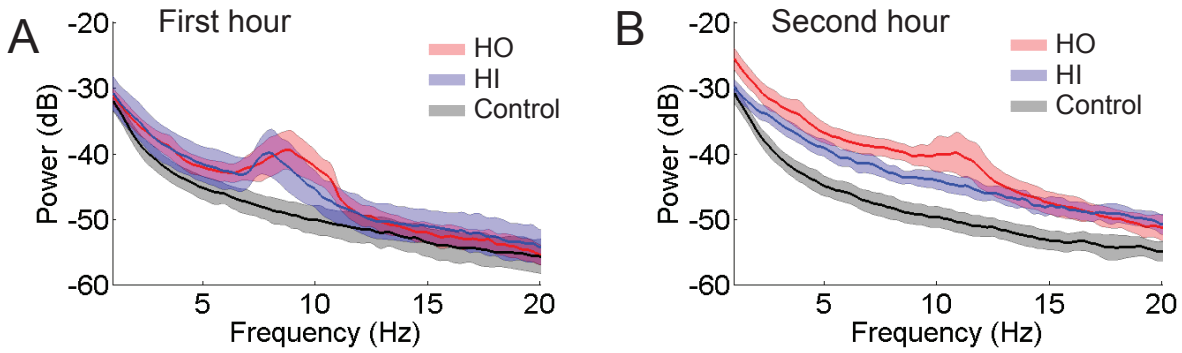

Figure 3.9. Frequency domain profile comparison of HI, Ha and untreated controls during first and second hours of treatment. During the first hour of treatment (A), both Ha and HI had increased power in the alpha (8-13 Hz) frequency band. During the second hour (B), power in HI was lower in delta and alpha frequency bands. The sharp increase in alpha power during the first hour is attenuated during the second hour of the treatment.

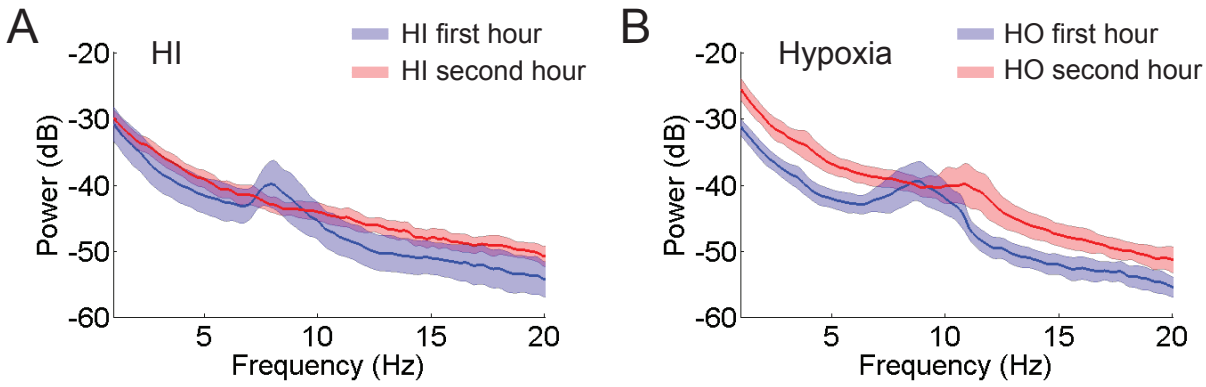

Figure 3.10. Frequency domain profile within HI and Ha groups. In the HI group, EEG power in the alpha band was attenuated during the second hour (A). The overall pattern of activity in Ha-treated animals showed an increase during the 2nd hour at all frequency bands (B). This increase was not present in HI-treated animals. In Ha-treated animals, activity in the delta and alpha frequency bands was increased during the second hour of treatment, and the peak alpha power shifted towards higher frequencies, from 9 to 11 Hz (B).

Table 3.1. Statistical analysis of event distribution. RMS power of seizures and background were compared between Ha- and HI-treated animals. Comparisons were conducted using Student's t-test when events were normally distributed and Mann-Whitney U test when events did not show a normal distribution (marked by *). RMS power was statistically different between HI and Ha groups, with most statistically significant values during the second hour of the treatment.

	Hypoxia			
	Background	All Seizures	Alpha Events	Delta Events
HI	**< 0.001**	**0.037**	**0.002**	**0.006**

	Hypoxia 1st hour			
	Background	All Seizures	Alpha Events	Delta Events
HI 1st hour	**< 0.001**	**0.03**	0.49	0.904*

	Hypoxia 2nd hour			
	Background	All Seizures	Alpha Events	Delta Events
HI 2nd hour	**< 0.001***	**0.001**	**.002***	**0.001**

CHAPTER 4

SUBACUTE BACKGROUND EEG ABNORMALITIES IN THE RAT MODEL OF NEONATAL HYPOXIC-ISCHEMIC ENCEPHALOPATHY ARE PREDICTIVE OF BRAIN DAMAGE

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Abstract

In this study we quantitatively examined subacute EEG in two models of neonatal seizures. We used single-channel EEG recordings to compare background EEG patterns in: (1) Hypoxia-ischemia (HI), which produced acute seizures with a brain lesion and was associated with the development of epilepsy and (2) Hypoxia alone (Ha), which caused seizures but was not associated with an obvious negative outcome. Using these models, we analyzed EEG recordings at 15 min, 6 h, 24 h, 48 h, 72 h and 96 h after the insult. We hypothesized that quantitative analyses of the EEG background and features of the electrographic seizures could differentiate between the insults of harmful versus benign etiologies. The presence of a lesion was associated with suppression of background activity in the beta and gamma frequency bands of the EEG. Additionally, detection of this background abnormality was superior to subacute seizures as a predictor of the presence of catastrophic brain injury (i.e. lesion), particularly when intermittent EEG recording protocols were used. Therefore, background suppression in these animal models is a potential biomarker for non-invasive, rapid detection of neonatal brain lesions.

Introduction

Perinatal brain injury and issues with treatment

Perinatal brain injuries are a significant cause of neurologic disorders such as epilepsy, cerebral palsy and intellectual disabilities. They are often a result of hypoxia-ischemia (HI) during intrapartum and postnatal periods (~3 in 2000

births) (Lynch et al., 2002). Unfortunately, the functional and structural deficits are usually detected after the acute seizures begin to occur. This is problematic, because seizures can cause a secondary injury in an already damaged brain (Wirrell et al., 2001; Moshe, 1998; Williams et al., 1992). Administering a therapy, such as hypothermia, as soon as possible is critical; however, therapies are often initiated too late, after both the primary and secondary injuries may have occurred. Background EEG abnormalities, such as suppression, have been reported to precede seizure activity (Williams et al., 1992; Levene, 1993), making them an excellent candidate for the development of an early marker of brain injury, when an intervention would be most valuable.

EEG in the acute and subacute periods

Multiple retrospective clinical studies suggest that the *subacute* spontaneous seizures that occur *after* the insult and the abnormalities of the background EEG can be predictive of a negative outcome (Legido et al., 1991; Tharp et al., 1989; Clancy et al., 1991; Mizrahi et al., 2000; Monod et al., 1972; Tharp et al., 1981; Holmes and Lombroso, 1993). Current monitoring protocols involve traditional EEG and amplitude-integrated EEG (aEEG). Unfortunately, these techniques require long epochs of recording and take a considerable amount of time to collect and analyze by a trained epileptologist, delaying their use for interventional screening. Simple, quantifiable and temporally sensitive biomarkers that could be used to screen neonates at risk of having an injury or to detect the optimal intervention window currently do not exist. By using animal

models of neonatal seizures, we aimed to investigate and quantify EEG features associated with a negative versus positive outcome, as defined by the presence of a cerebral lesion.

Experimental approach: background EEG in subacute periods of HI- and Ha-treated animals

Neither background EEG nor subacute electrographic seizures have been widely investigated using animal models. Instead, the focus in the animal models has been on treating *acute* seizures that occur *during* the insult as the main outcome predictor and therapeutic target for intervention (Dzhala et al., 2005; Raol et al., 2009; Aujla et al., 2009). Development of animal models that focus on the subacute (i.e., minutes to hours after the insult) seizures and background abnormalities is a critical step in identifying electrographic features that could be used for early detection of negative outcomes and subsequent interventions. In this study, we used two animal models – HI and Ha in P7 rat pups. Ha induced robust electrographic and clinical seizures with no resulting brain damage, while HI resulted in acute seizures and a unilateral lesion in cortex, thalamus and hippocampus (Rice et al., 1981). We followed the administration of HI and Ha with serial EEG recordings and quantitatively analyzed subacute background EEG. We hypothesized that with quantitative analysis of the EEG background and ictal features, we could differentiate between insults of harmful and benign etiologies.

Materials and methods

Animals

All surgical procedures were performed under protocols approved by the University of Utah Animal Care and Use Committee. Untimed pregnant Sprague-Dawley adult female rats were received from Charles-River (Wilmington, MA). Pups were delivered in our animal facility on average 1 week from the arrival of the pregnant female. Litter size varied from 8 to 12 pups. Rat pups were housed with the dam and littermates over the course of the study (i.e., before and after they were implanted with telemetry).

Telemetry system

A proprietary miniature telemetry system, which was designed in-house, was used in this study. The system consisted of a dome-shaped transmitter 10 mm in diameter by 10 mm high, which weighed 1 g. The transmitter was designed to acquire 1 channel of EEG configured for differential recording from two electrodes. The EEG signal was amplified at the transmitter, which was wirelessly capacitive-coupled to the receiver base. The signal was then digitized with a Biopac MP150 analog-to-digital converter and recorded to the computer. The telemetry was bandwidth-limited to recording signals in the 0.1 – 120 Hz frequency range.

Surgical Implantation of the telemetry

Postnatal day 6 (P6) rat pups were implanted with the wireless transmitter. All surgical tools were autoclaved and their sterility was maintained with 70% ethanol. During the surgical procedure, animals were anesthetized with 4% isoflurane (MWI Veterinary Supply, Meridian, ID), and maintained at 2% over the course of the procedure. Animals were stabilized in a stereotaxic frame with small-animal ear bars (David Kopf Instruments). Rostro-caudal incision was made with a scalpel. Skin was then clamped with hemostats to gain access to the surgical field, pereosteum was scraped with a sterile cotton swap, and surface bleeding was controlled with a low temperature cautery pen (Bovie Medical, Clearwater, FL). A dental drill with a 0.7 mm burr (Fine Science Tools) was used to drill two burr holes for electrode placement. The holes were positioned 2 mm lateral from midline of the skull, separated by 2 mm anterior-posterior above the hemisphere that is ipsilateral to the ligated carotid artery. Electrodes were trimmed to the depth corresponding to the level of the dura (variable length due to variation in the shape of the transmitters) and were inserted into the burr holes. The transmitter was stabilized in place with cyanoacrylate gel compound (Loctite 454) with accelerator (Loctite 7452). Additional cyanoacrylate gel was applied around the transmitter to maximize the surface area for stable adhesion. Anesthesia was terminated and the animals were injected with 0.5 mL lactated Ringers (sub-cutaneous) and superficially treated with local anesthetic (Marcaine). Animals were allowed to recover for 24 h prior to administration of HI/Hypoxia.

Hypoxia-ischemia

The HI procedure, using the modified Levine's method (Levine, 1960), was performed on P7 rat pups that were previously implanted with telemetry units at P6. Animals were anesthetized with 2% isoflurane (MWI Veterinary Supply, Meridian, ID). A 2-cm incision was made in the animal's neck, and the right common carotid artery was exposed by blunt dissection, clamped with micro aneurism clamps and cauterized with a low temperature cautery pen. Skin was sutured and the animals were allowed to recover for 2 h with the dam. The entire duration of the procedure was 3-5 min. Following a recovery period, animals were placed in a custom-designed treatment chamber. The chamber consisted of clear acrylic housing with an aluminum platform, on which three receiver bases were placed. The temperature in the chamber was regulated with a feedback-controlled proportion-integrate-derivative (PID) temperature controller with a thermocouple. Temperature was held at 37°C and crosschecked using an independent thermocouple (Vernier Instruments, Beaverton, OR) during the treatment and monitoring periods. Each animal was placed in an individual sealed acrylic treatment chamber and hypoxia mixture (8% oxygen, balance nitrogen) was administered at a 1 L/min rate into each chamber through a manifold. Animals were exposed to hypoxia for 120 min. Following treatment, they were allowed to recover for 15 min, injected with 0.5 mL Lactated Ringer's sub-cutaneous and placed back into the recording chamber with normal air for monitoring. Carotid arteries were left intact in the hypoxia-treated animals, and

controls were placed in the treatment chambers at the same temperature with normal air.

Monitoring

EEG was monitored in the treated animals over 6 recording periods: 15 min, 6 h, 24 h, 48 h, 72 h and 96 h after administration of HI or hypoxia. For monitoring, animals were placed in the previously described temperature-controlled chamber and EEG was recorded for 2 h. After each monitoring period, the animals were injected with 0.5 mL Lactated Ringer's solution sub-cutaneous to prevent dehydration prior to returning to the dam.

Quantitative EEG analysis

EEG recorded from rat pups during monitoring periods was analyzed using quantitative measures. The first 30-min epochs of each recording period were separated and transmitter-induced "drop-out" artifacts were manually removed using the Acknowledge 4.1.1 (Biopac, Golete, CA) software. The data files were then converted into MATLAB format and analysis was performed using MATLAB software by Mathworks (Natick, MA). Fast Fourier Transforms (FFTs) were performed to analyze EEG data in the frequency domain from 0.1 to 60 Hz. Power spectral densities (PSDs) were estimated from the FFT using the 2048 Hann-window segments based on the Welch method and normalized by 10log10 (PSD). Mean power levels at all frequencies in the 0.1 to 60 Hz range were plotted with 95% confidence intervals. To compute the integrated power in each

band, the EEG bands were defined as delta (0.1-4 Hz), theta (4-8 Hz), alpha (8-13 Hz), beta (13-30 Hz) and gamma (30-60 Hz) and power from PSD was integrated in these ranges. The integrated power for each band was averaged across animals and plotted as a function of recording period group.

Statistical analysis

Comparisons were made between HI-treated group and control, and Ha-treated group and control using paired t-tests with MATLAB (Natick, MA).

Results

Background suppression: qualitative observations of the early vs. late stages of the injury

In the HI-treated animals, background suppression became evident immediately after termination of the hypoxia-mixture administration. Recordings of 2-h EEG epochs were conducted 15 min, 6 h, 24 h, 48 h, 72 h, 96 h after administration of HI (and at equivalent time points in controls), and 30 min of background EEG was analyzed. Background suppression manifested itself qualitatively in the EEG as a signal of reduced amplitude. In the recordings collected 15 min after administration of the insult, the EEG ranged from isoelectric to a nearly isoelectric signal with small-amplitude spikes and waves present in the recording (Figure 4.1). In the later recording groups, the signal started to “recover” to more normal amplitude, but the EEG waveforms were different than those in the control group. Large spike-wave events in the delta

range were first to recover in the 24-h group. This pattern continued over the next 3 days with delta and theta activity recovering to the levels of control, but higher frequency activity in the HI group remained profoundly suppressed, and never recovered during our monitoring periods. EEG signals were quantified by integrating power in each of the defined EEG frequency bands: delta 0.1-4 Hz; theta 4-8 Hz; alpha 8-13 Hz; beta 13-30 Hz and gamma 30-60 Hz. Statistically significant suppression of the signals was present in all of the EEG bands when examined in both frequency (Figure 4.2) and temporal domains (Figure 4.3) at: delta, theta, alpha, beta and gamma ($p<0.001$ for all bands). When integrated power was measured for the 6-h group (Figures 4.3), the low-frequency bands (i.e., delta and theta) showed recovery with no significant difference between control (n=12) and HI (n=9) groups. However, statistically significant suppression of power was present in the alpha ($p=0.015$), beta ($p<0.001$) and gamma ($p<0.001$) bands. In the 24-h group (Figures 4.2C, 4.3), the progressive recovery continued, with no significant difference in the delta band, but significant differences were still present in the theta ($p=0.036$), alpha ($p=0.035$), beta ($p<0.01$), and gamma ($p<0.001$) bands. The low-frequency bands (i.e., delta, theta and alpha) had recovered by 48 h, and showed no statistically significant difference between HI and control groups; however, suppression was still present in the beta ($p<0.001$) and gamma ($p<0.001$) bands. In the 72-h posttreatment group, no statistically significant difference was present in the delta band, but integrated power in the alpha ($p = 0.003$), theta ($p=0.014$), beta ($p<0.001$) and gamma ($p<0.001$) bands remained different. Significant differences were still

present in the alpha ($p=0.016$), beta ($p<0.001$) and gamma ($p=0.002$) bands at 96 h. The differences were qualitatively evident in the mean power spectral density graphs when plotted with 95% confidence intervals (Figure 4.2). These findings supported the hypothesis that quantifiable differences in background EEG were present in the HI group when compared with control animals, and that these differences persisted for several days after the HI insult.

Background EEG activity in the Ha group

In the Ha group ($n=12$), a transient suppression effect on EEG background was detected in the 15-min group (Figure 4.5C, D) for the beta ($p=0.044$) and alpha ($p=0.024$) bands when integrated power was analyzed as before with the HI group. This effect was very transient, however, and did not persist into the later time points. When examined in the frequency domain on the logarithmic scale with calculated mean and 95% confidence intervals, the early suppression effect was not apparent (Figure 4.4A). The background EEG appeared to be completely recovered 6 h after the Ha treatment. When examined qualitatively, no differences in the background signal could be detected between the control and Ha groups. Therefore, following Ha treatment, a transient background abnormality may have been present early in the 15-min group; but it recovered shortly after the insult.

Background suppression precedes seizures

In the HI group, *subacute* seizures were recorded in 3/9 animals. These seizures had different waveforms than the *acute* seizures that occurred during the administration of hypoxia in all animals in both HI and Ha groups (Figure 4.6). In 2/3 animals with seizures, the subacute seizures occurred in the 15-min group, and in 1 animal in the 96-h group. Seizure duration in the 15-min group was 169 sec and 90 sec, and in the 96-h group seizures occurred in a cluster of two with durations of 33 and 36 sec. The seizures in the 96-h group were accompanied by ictal spike-wave activity. No subacute seizures were detected in the Ha or control groups. In the animals with seizures, background suppression in the 15-min group appeared to precede the occurrence of the seizures. To quantitatively confirm this, 10-min portions of the background EEG signal were analyzed using mean power spectral density with 95% confidence interval-plots and mean integrated EEG power. Signals from HI-treated animals with seizures (n = 3) and HI-treated animals with no seizures (n = 3) were compared. The difference was apparent on the power spectral density plot, with the signals of those HI-treated animals that had seizures overlapping the signals of the HI-treated animals where no seizures were recorded. Both of these groups were significantly different from the control group (Figure 4.7). The same effect could be detected using integrated power analysis when EEG bands were grouped into low-frequency bands of 0.1-13 Hz (i.e., delta, theta and alpha) and high-frequency bands of 13-60 Hz (i.e., beta and gamma). In the low-frequency bands, statistically significant differences were detected between HI with *seizures*

compared to control (p=0.05) and HI with *no seizures* compared to control (p=0.04). A similar effect was detected in the high frequency EEG bands: HI *with seizures* vs. control (p=0.043) and HI with *no seizures* vs. control (p=0.039). No statistically significant difference was found when HI with seizures was compared to HI with no seizures (p=0.542). It is likely that seizures occurred in more HI-treated animals in this study, but were not detected because of the lack of continuous 24-h/day EEG monitoring.

Temporal sensitivity: detection of differences with short recording epochs

In order to determine the minimum recording epoch required to detect the difference in the background EEG, we used shorter epochs than those in the main analysis. For this, 10-min and 1-min epochs were evaluated, and the temporal sensitivity was examined at 15-min and 48-h in the HI-treated (n=9) and control (n=12) groups (Figure 4.8). The first 10-min and the first 1-min of recordings were selected from each group, and then mean power spectral densities with 95% confidence intervals were plotted. Due to the highly suppressed background EEG activity after HI, 1-min recording epochs were sufficient to detect the differences between HI and control in the 15-min group (Figure 4.8A). In the 48-h group, a 1-min recording epoch showed a significant effect with the 10-min epoch in the high-frequency EEG bands (i.e. beta, gamma). Using a 1-min epoch, significant differences were observed in the beta band; however, due to variability in the signal, the ability to detect the differences

was lost. Thus, it appeared that in the early recording group (i.e., 15 min after the insult), a 1-min recording epoch was sufficient to quantitatively detect the difference between HI and control. However, in the later recording group (48 h), a 10-min epoch appeared to be required for differentiation between the two conditions (Figure 4.8B).

Discussion

Suppression of power in the beta and gamma bands

Outcome predictors are extremely important in both clinical studies as well as animal models designed to mirror the human disease processes. Several clinical reports have described seizures and abnormalities of the background EEG as effective outcome predictors (Holmes and Lombroso, 1991; Clancy and Legido, 1991; Tharp et al, 1989; Legido et al., 1991; Shinnar et al., 1990; Pezzani et al., 1986; Korotchikova et al., 2011; Tharp et al., 1981; Monod et al., 1972). These outcomes ranged from normal to negative outcomes, including encephalopathy, epilepsy and cerebral palsy. Based on previous studies in our laboratory that found the presence of the HI-induced brain lesion to be required for the development of epilepsy (Kadam et al., 2010), we defined a negative outcome as the presence of a lesion at 96 h after administration of HI. The data presented here indicate that all animals with HI-induced lesions had quantifiable EEG power suppression in the beta and gamma bands. Therefore, we propose that background suppression in the beta and gamma bands could be used as a predictor for the presence of a lesion. Combined with previous data from our

laboratory, this finding suggests that abnormalities in background EEG have the potential to be used as an early biomarker of subsequent epileptogenesis in this animal model. Studies that wish to examine the development of epilepsy require considerable time and material investment due to the lengthy disease process and requirement for chronic monitoring. Using early detectable outcome predictors, such as the ones described in this manuscript, could enable lower cost designs for screening of therapies while recommending closer monitoring of the disease process that would potentially translate into better management of the condition clinically.

Administration of the insult: approximating the clinical scenario

A major advantage of the experimental timeline used in this study is its similarity to the clinical scenarios. Most animal models of neonatal neurologic conditions such as neonatal seizures have used the period *during* the administration of the insult (i.e., hypoxia, HI, kainate treatment) as a time-frame for interventional testing, often utilizing pretreatment protocols (Raol et al., 2009; Dzhala et al., 2005; Aujla et al., 2009; Koh and Jensen, 2001). This scenario often does not represent the clinical setting, where patients are evaluated *after* the insult has occurred. Injuries such as HI often progress rapidly, with various changes in such factors such as metabolism, inflammation, and phases of cell death occurring at different time points (Riezzo et al., 2010; Hossain, 2005). Therefore, it is plausible that interventions that have been tested and found effective in an experiment using a pre-treatment protocol (i.e. *before* the insult)

might be ineffective when applied in a more clinically-relevant situation (i.e., *after* the insult). This study examined background EEG minutes, hours and days after the insult had occurred. The data obtained here suggest that this model and these analyses could be used as a baseline for testing the effectiveness of various therapeutic interventions. This approach would enable therapeutic testing using animal models in the subacute period after the injury, the time when a clinical intervention is most likely to be used in a clinical situation.

EEG frequency response: a candidate biomarker for determining the phase of the injury

Changes and abnormalities in the underlying brain structure and physiological function can be correlated with the changes in the EEG. Thalamic lesions have been described to induce delta waves in cats (Gloor et al., 1977), and quantifiable EEG suppression was reported after HI in fetal sheep following HI (Williams et al., 1992). In the present experiments, we found two distinct EEG power and frequency profiles based on the timing of the recording period after the insult. The first EEG profile was characterized by suppression at all frequency bands (0.1-60 Hz) and was detected immediately (minutes) after the insult. Over the course of 6 h, the first profile evolved into the second profile - one that was characterized by normal power in the low-frequency bands (delta, theta) and suppressed power in the high-frequency bands (beta, gamma). Suppression in the alpha band was not a reliable indicator, because it only appeared in some groups and was not a consistent finding. We propose that these EEG profiles

could be indicating two phases of the injury. Suppression of EEG caused by spreading depolarization during ischemia has been previously recorded in both human and animal models (Drenckhahn et al., 2012; Dreier et al., 2009; Dohmen et al., 2008; Mies et al., 1993). Thus, it is plausible that during the first phase of the injury (suppressed low-frequency bands), the peri-infarct areas of the brain remain in depolarization-inactivation, producing an isoelectric EEG pattern. After a delay, however, this area recovered sufficiently to restore the power in the low-frequency bands while the power in the high-frequency bands (i.e., beta and gamma) remained suppressed, perhaps due to the underlying damage. To test this hypothesis directly using our animal model, the telemetry device would have to be modified to be able to record DC shifts in the EEG signal. Without establishing the direct mechanism, we propose that time-dependent changes in the power and frequency of the EEG signal could be used to determine the progression or the phase of the injury (i.e., time after insult). We hope to further develop the concept of a temporal relationship between the injury and EEG signal as a vehicle for identifying electrographic biomarkers for the various stages of brain damage.

Background suppression: a candidate strategy for screening and diagnostics

An important issue in the field of perinatal stroke is the relationship between acute seizures and the underlying brain damage. Controversy exists whether seizures alone damage the neonatal brain (Camfield, 1999; Wasterlain,

1999). Numerous studies suggest that while the neonatal brain may be resistant to acute seizures, if the seizures occur in a brain where an underlying lesion is already present, they further damage the brain and aggravate the injury, thereby causing a secondary injury (Wirrell, 2001; Germano et al., 1996; Germano et al., 1998; Moshe, 1998; Shinnar et al., 1990). This is particularly critical in diagnosis and treatment of perinatal stroke. Acute seizures follow an insult in the first 72 h after the event, often triggering the initial evaluation for stroke (Nelson and Lynch, 2004). The timing of the evaluation for stroke after the seizures is problematic; only after the initial infarct and the secondary injury due to seizures would the diagnosis and subsequent intervention occur. Clearly, developing a screening or diagnostic strategy to detect a problem before secondary injury occurs would be beneficial. With the intermittent monitoring periods used in this study's design, we found that acute seizures were present in three animals after HI. While we expected to detect some seizures during our monitoring periods, we found that in animals where these events occurred, background suppression always *preceded* the seizures in the early recording epoch. The suppression was present in the animals where seizures were recorded in both short-term (minutes) and long-term (days) periods after the HI. Thus, the presence of background suppression may have been predictive of the acute seizures in these animals. This hypothesis would have to be further tested by utilizing continuous "24-7" monitoring protocols. We propose that the presence of EEG suppression could be used as an early warning sign for HI encephalopathy. Because suppression was relatively easy to quantify and required only one channel per

hemisphere to record, it could potentially be used as a screening strategy for identifying perinatal stroke early, before secondary injury by acute seizures occurs.

Background suppression present in the HI group, but not hypoxia group

Animals in both HI and Ha groups undergo robust electrographic and behavioral seizures *during* the administration of the insult. The EEG profile *after* the insult was very different between the two groups. The background EEG profile of the Ha-treated group was similar to the one in the control group (no brain damage), while the HI group displayed background suppression indicating that the lesion was present. One of the current controversies in the field of acquired epilepsy is whether neuronal death is necessary for acquired epileptogenesis, or whether seizures alone are sufficient (Kadam et al., 2010; Baram et al., 2011; Rakhade et al., 2011; Dudek et al., 2011). While the data presented here did not directly address this controversy, they have suggested that background suppression after an insult is indicative of the neuronal death-dependent pathway of epileptogenesis. Because the background activity in the Ha group was similar to that in control, it is difficult to infer the outcome in this group using the data analysis techniques used here. Several clinical studies report that EEG abnormalities, such as suppression in the background patterns, predict not only epileptogenesis, but also conditions such as cerebral palsy and intellectual disabilities, which require frank brain damage (Clancy and Legido, 1991; Cummins et al., 1993). Thus, HI likely models the worst-case scenario of

perinatal brain injury, while Ha models a milder type of injury with no brain damage. We propose that if translated into a clinical setting, our strategy - which relies on power and frequency of the background EEG - would be most effective in the cases that would benefit from an early intervention. However, it would likely lack sensitivity to predict outcome in more mild types of injuries that do not involve neuronal death.

Electrographic biomarkers: optimizing EEG as a tool for rapid detection of HIE abnormalities

Electrophysiological tools such as EEG have excellent temporal resolution. Unfortunately, it is often not utilized as a temporally-sensitive tool. Because a highly trained expert normally analyzes EEG using qualitative techniques, hours often pass between EEG acquisition, analysis and the implementation of an intervention based on the EEG findings. While this approach is adequate for detecting and treating seizures associated with chronic epilepsy, a timely intervention is critical in patients with stroke. The timing of the intervention may potentially benefit from a rapid quantitative EEG analysis. Based on our data, we propose that background EEG analyzed with FFTs could provide such an approach. We analyzed 30-min, 10-min and 1-min recording epochs of background EEG. Shortly after the HIE insult, all of these recording epochs provide an excellent profile with significant differences. Thus, in the HI model used here, 1-min of EEG recording was sufficient to determine whether the animal was subjected to HI and in the process of developing a catastrophic

brain lesion. At later recording time points, 1-min epochs remained significant, but the analysis approached marginal levels of resolution between HI and control groups. At these time points, a 10-min epoch provided a better predictive value for the presence of HI. It is arguable that at earlier time points, temporal resolution is more important, as it would shorten the time required for detection of an abnormality, leaving more time for further evaluation and administration of intervention. While it is not clear whether these strategies can be translated to human use, quantitative analysis of epochs as short as 1 min would significantly highlight the use of EEG as a temporally sensitive tool. A short-duration recording would not necessarily provide information about etiology of the injury, but it would be helpful in determining the presence of the abnormality. Further testing with retrospective approaches in human patients will be required to confirm the feasibility of this concept.

Conclusions

In this study, we examined abnormalities of background EEG, a concept originally described in the clinical literature; we applied these concepts to animal models of neonatal seizures and stroke. We found that multi-frequency suppression of background EEG could be used as a biomarker for early detection of perinatal stroke, as early as 15 min after the insult. Additionally, suppression of EEG power in the beta and gamma bands could be used to detect a lesion in the brain as late as 96 h after the insult. These approaches were temporally sensitive, requiring as little as 1 min of EEG recording during

early stages (15 min after insult) and 10 min in the late stages (48 h after insult) of HI-induced brain injury. Additionally, the abnormalities in the background EEG could be recorded before the appearance of acute seizures, enabling identification of an injury that has not yet been aggravated by the seizures. These concepts, which were developed using animal models, may be applicable to early screening for perinatal stroke and identification of individuals that would benefit the most from a prompt intervention; studies will soon be initiated to examine this issue in more detail.

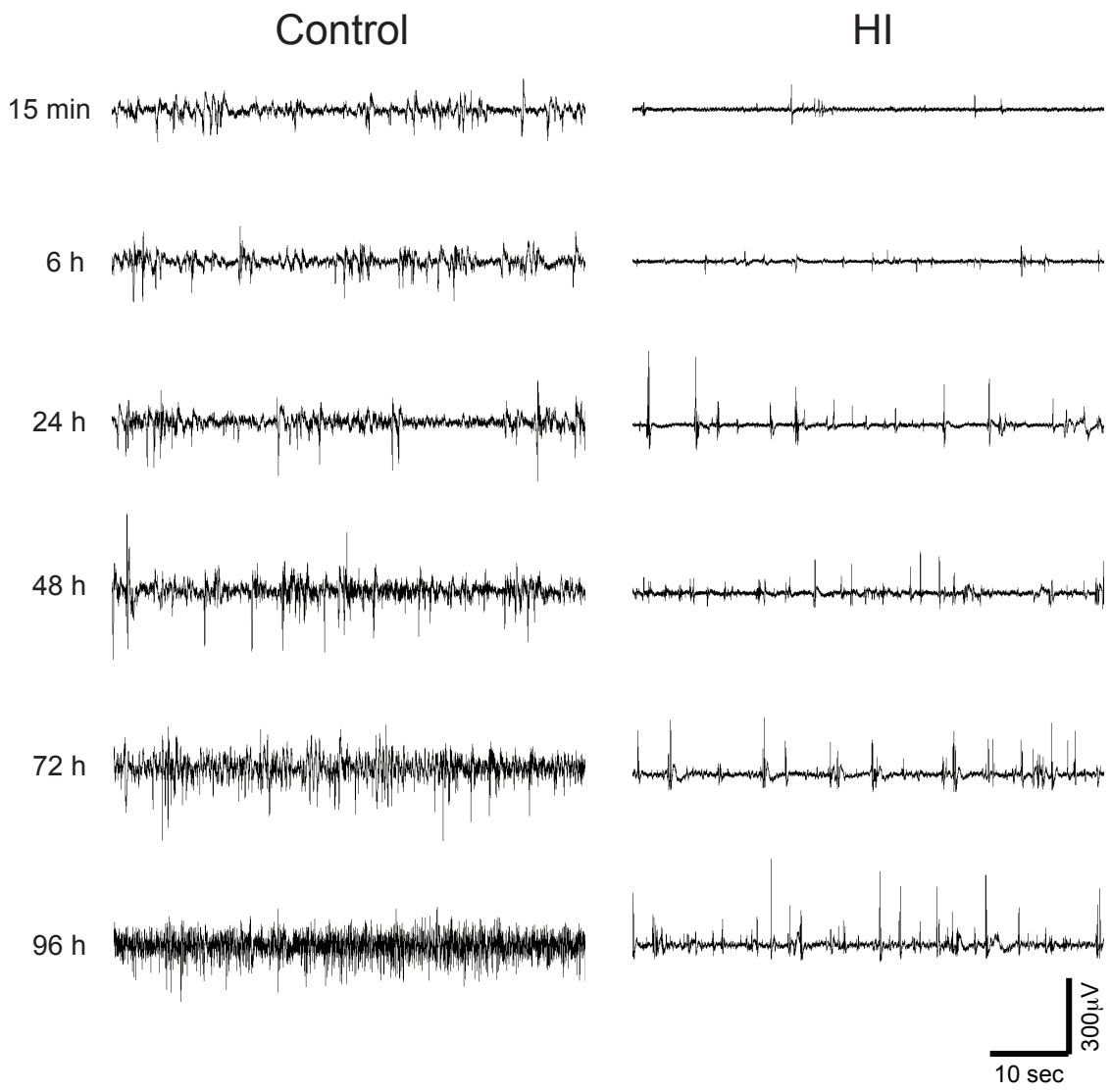


Figure 4.1: Raw EEG traces from control and HI-treated animals in the sub-acute period. Background EEG was recorded from control and experimental groups 15 min, 6 h, 24 h, 48 h, 72 h and 96 h after administration of HI or at equivalent time in the controls. Control group showed age-dependent maturation of the EEG signal, while the signal in HI group remained suppressed and lacked maturation-dependent increases in high-frequency (i.e., beta and gamma) activity.

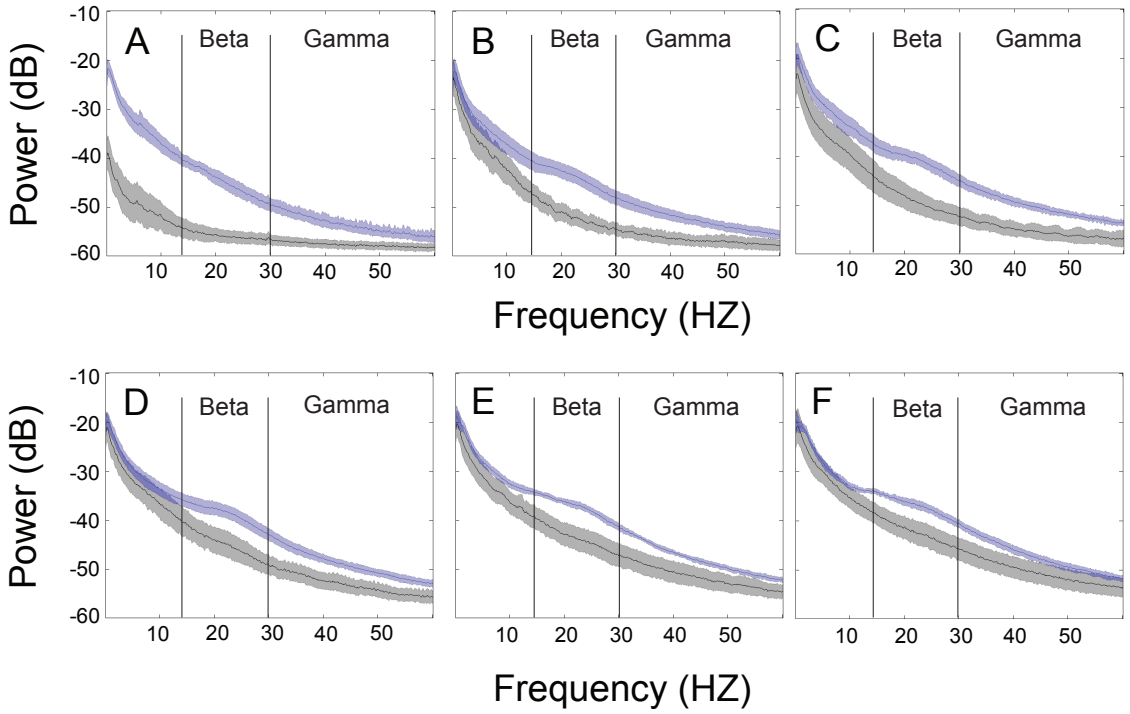


Figure 4.2: Power spectral densities of background EEG from HI and control animals as a function of time after the insult. PSDs were obtained from HI and control animals. Mean PSD was plotted with 95% confidence intervals for HI (black) and controls (blue) in each of the recorded groups (A-F). In the 15 min group (A), the HI animals show background suppression at all frequencies (A; 0.1-60 Hz). Over time, in the 6 h (B), 24 h (C), 48 h (D), 72 h (E) and 96 h (E), the low-frequency EEG bands (i.e., delta, theta, alpha) appeared to undergo a recovery, but high-frequency EEG bands (beta, gamma) remained significantly suppressed. Control animals showed a normal maturation profile with high-frequency EEG bands increasing in power as a function of age.

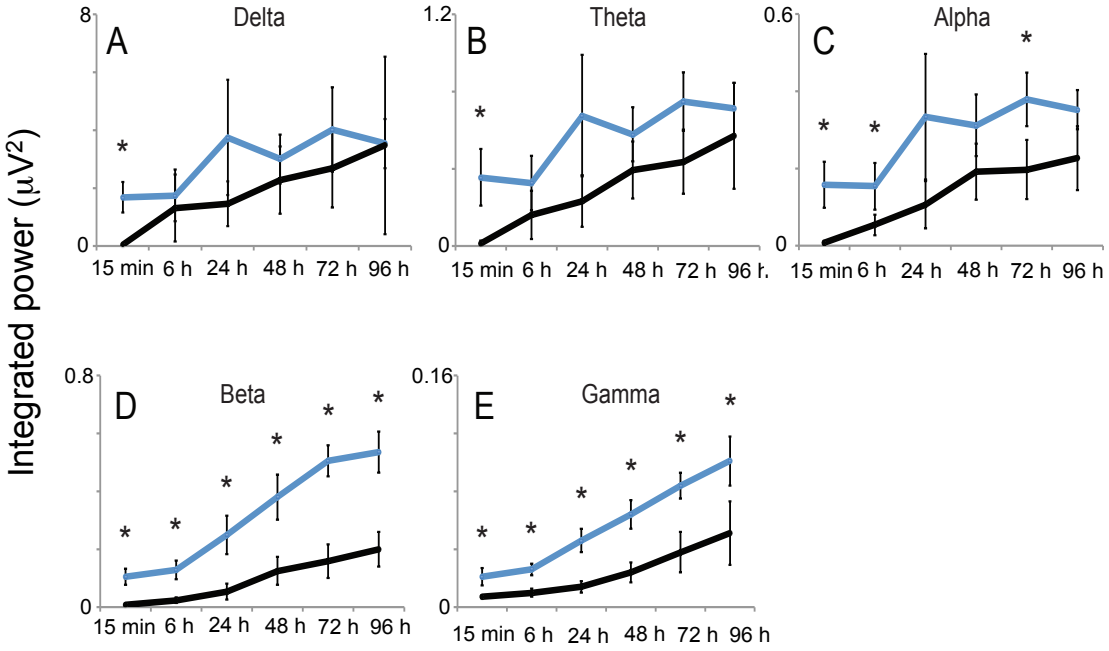


Figure 4.3: Integrated power in EEG bands in HI and control animals. Power was integrated in each of the frequency bands and plotted as a function of time after treatment. Integrated power was compared between HI (black) and controls (blue) using t-test. In the 15-min group, significant differences were apparent in all of the frequency bands. As the animals recovered from HI, statistically significant differences persisted in the beta and gamma bands (D, E), and low-frequency bands (i.e., delta and theta) were no longer indicative of the difference between the two groups. Significant differences between HI and controls were detected in the alpha band in the 15-min, 6-h, and 72-h groups (C). Error bars = standard deviation.

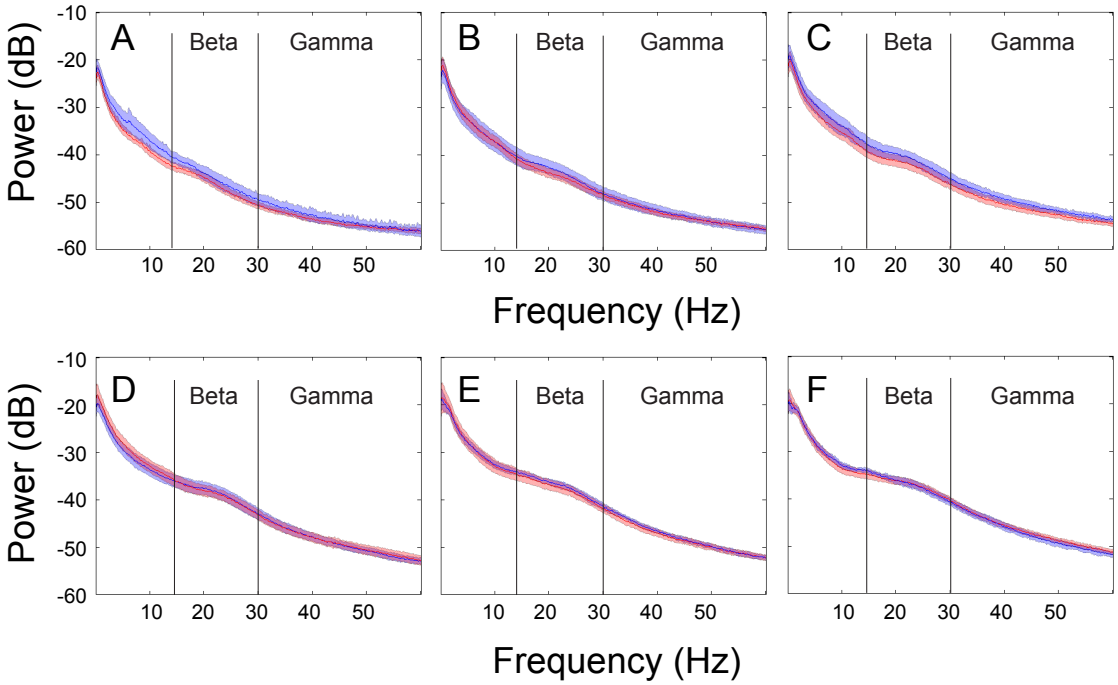


Figure 4.4: Power spectral densities of the background EEG from Ha-treated and control animals as a function of time after the insult. PSDs were estimated from Ha-treated and control animals. Mean PSD was plotted with 95% confidence intervals for Ha (red) and control (blue) animals in recordings conducted 15 min (A), 6 h (B), 24 h (C), 48 h (D) 72 h (E) and 96 h (F) after the insult. With this method of analysis, there were no apparent differences between Ha and control groups. Animals appeared to recover from the Ha insult immediately and did not develop any abnormalities in the background EEG.

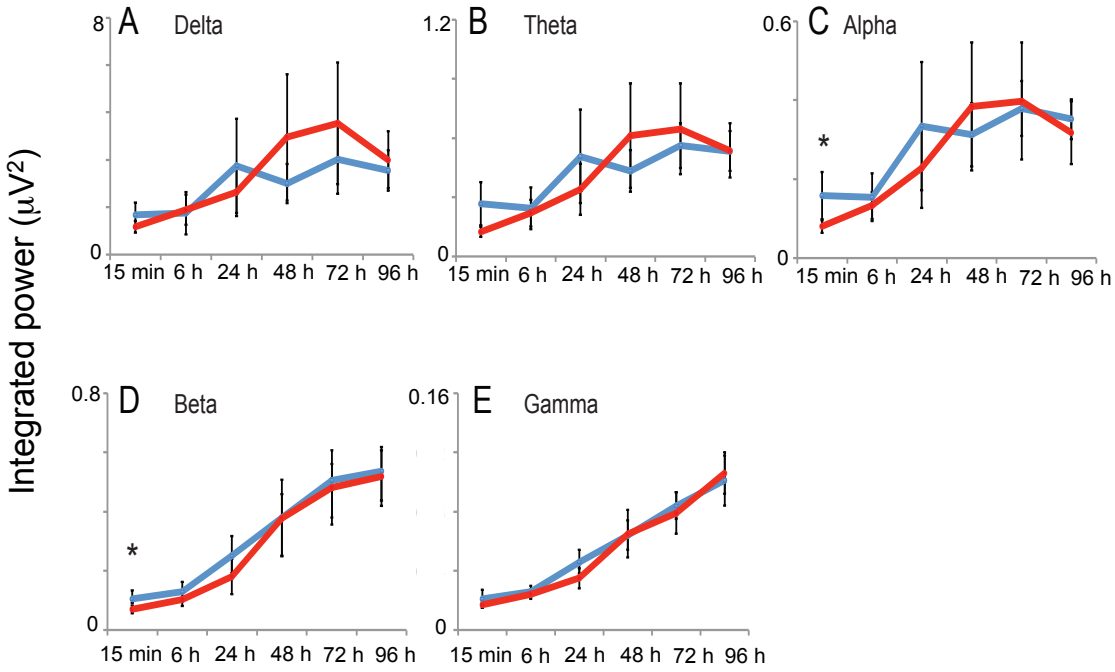


Figure 4.5: Integrated power in the EEG bands in the Ha-treated and control animals. Power was integrated in each of the EEG frequency bands and plotted as a function of time after treatment. Using this method, a statistically significantly lower power was detected in the alpha and beta bands of Ha-treated animals in the 15-min group (C, D). The power appeared to recover and stay at recovered levels during the subsequent recording sessions. Ha (red), control (blue).

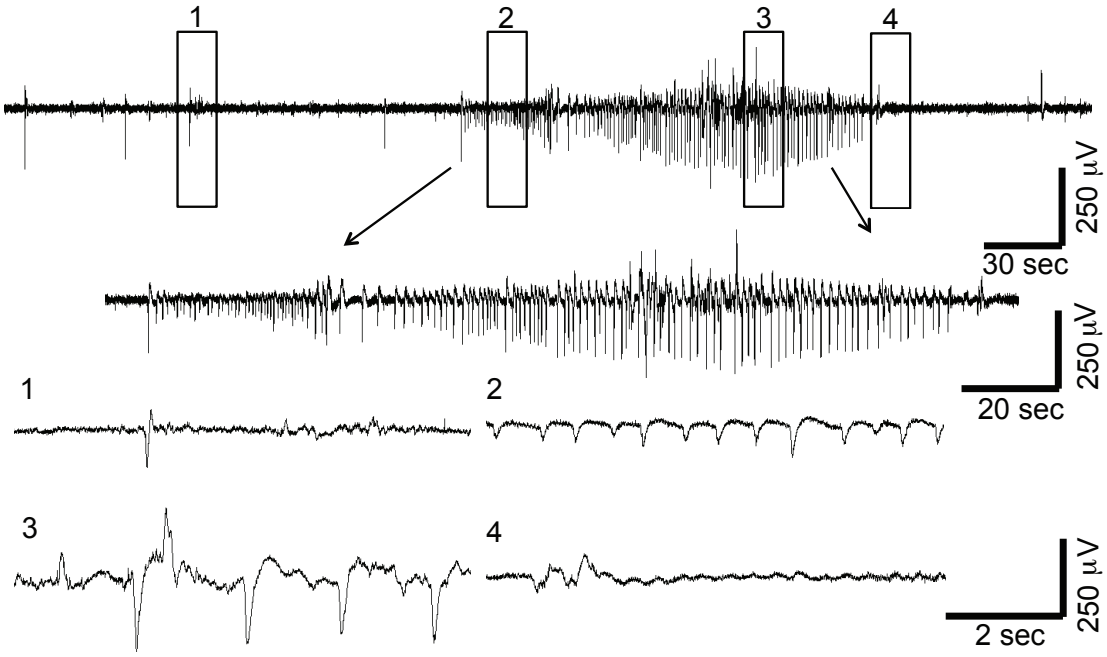


Figure 4.6: Subacute seizure in the HI-treated group. Subacute seizures were recorded in 3/10 animals in the HI group. The seizures were similar to classic seizure patterns, consisting of large-amplitude spike-wave activity (1-4).

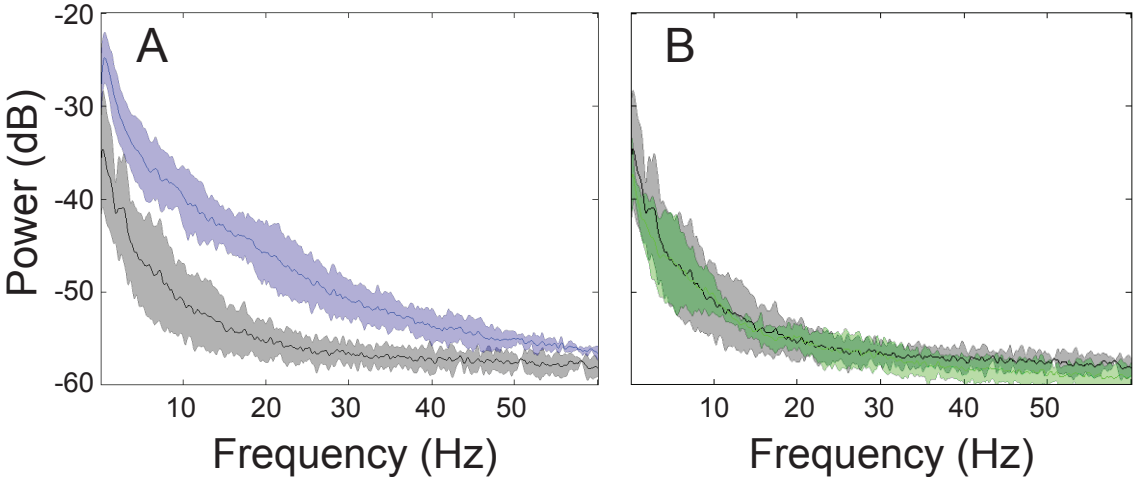


Figure 4.7: Background suppression precedes seizures in the animals where subacute seizures were detected. Ten-min epochs of background EEG were analyzed by estimating PSD in HI animals with subacute seizures (n=3), HI animals with no subacute seizures (n=3) and animals from the control group (n=3) in the 15-min group. Mean PSDs were plotted with 95% confidence intervals for each group – HI with seizures (black), HI with no seizures (green) and controls (blue). Significant background suppression was detected in HI-treated animals in both animals with and without subacute seizures (A, B).

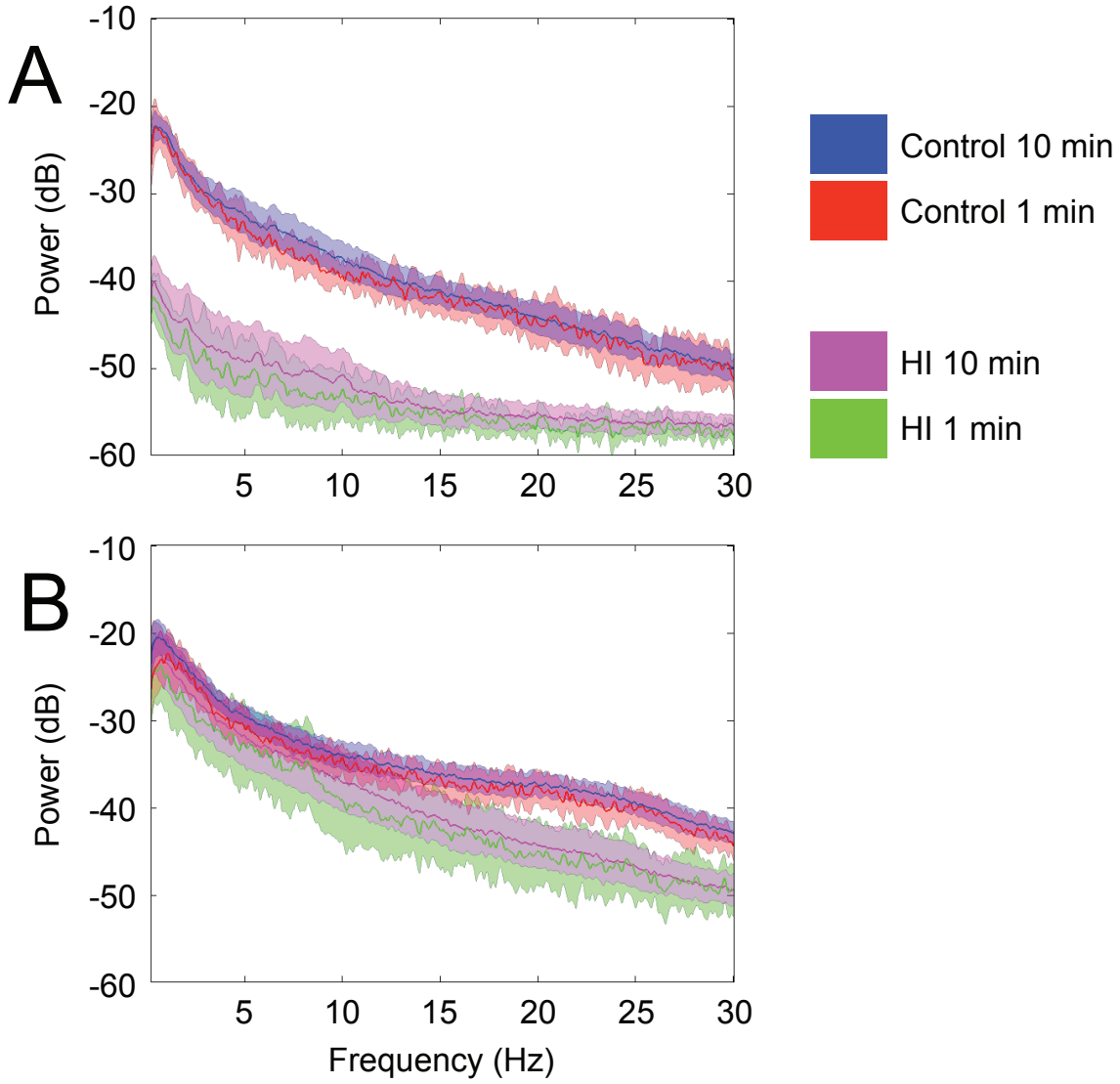


Figure 8: Temporal sensitivity of the background EEG analysis. To test whether our analysis technique has sensitivity to differentiate between HI and controls using shorter recording epochs, 1-min and 10-min long background EEG traces from HI (10 min – magenta; 1 min – green) and control (10 min – blue; 1 min – red) were tested. PSDs were estimated and means were plotted with 95% confidence intervals. Data was obtained from the 15-min group to test for temporal resolution short-term and 48-h group to test resolution long-term.

CHAPTER 5

DISCUSSION

The fundamental problem addressed by this research

An important clinical problem in neonatology and pediatric neurology is the lack of reliable diagnostic tests that can rapidly and reliably detect HIE and other acute brain insults that occur during and after birth; because EEG requires trained technicians and neurologists, acquisition and interpretation of EEG data often takes many hours. EEG has been routinely applied in clinical practice, but generally the conclusions from EEG as an outcome measure are derived retrospectively. Little work has been done in animal models to develop electrographic measures that could be used prospectively to predict long-term outcome. This is particularly true in research that utilizes rodents, a low-cost standard of most animal research. Using EEG in neonatal rodent models has been extremely difficult due to technical limitations, and the first goal of this research was to address these technical problems. Once most of the technical obstacles were overcome, the long-term goal of this research became approachable: *to use neonatal EEG to identify* – and then, ultimately, to predict (1) the insults that occur with neonatal seizures but result in little or no neuronal

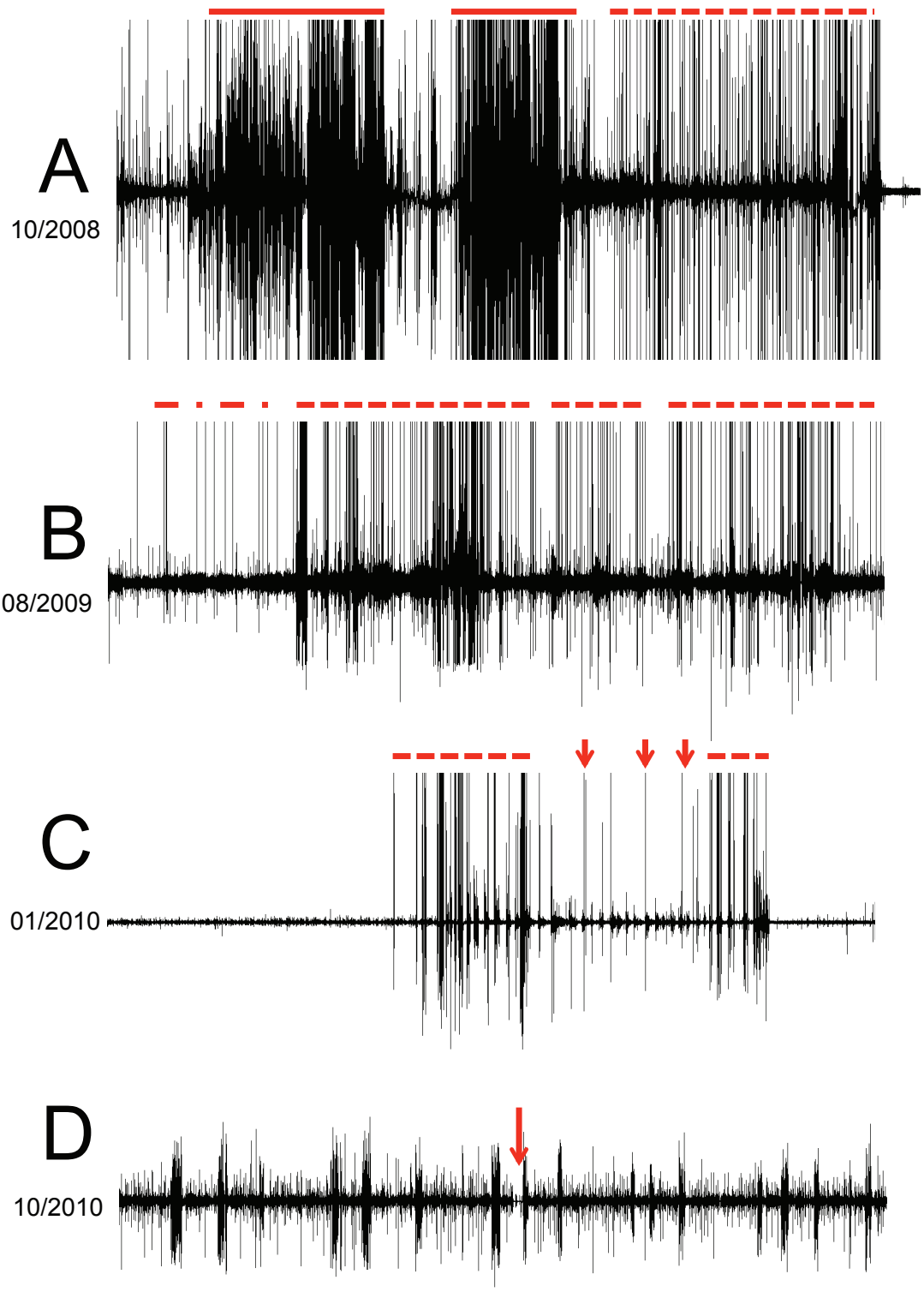
death (e.g., a relatively benign outcome) and (2) those insults that will cause catastrophic brain damage (a negative outcome, such as cerebral palsy and epilepsy). The main aim of this particular research project was to develop the ability to differentiate between neonatal seizures that occur in a rodent model of catastrophic HIE (i.e., hypoxia-ischemia [HI]) and another model with neonatal seizures that results in no obvious neuronal death in rat pups (i.e., hypoxia alone [Ha]). This aim was accomplished by analyzing the background properties of neonatal EEG recordings; the findings from this work may be useful in the development of biomarkers for early detection of HIE.

Development of the telemetry system enabled this research

At the start of this research project, the tools and instrumentation that could be used for high-quality EEG recordings in rat pups were not commercially available. After extensive modifications to attempt to adapt a wired system for use in rat pups, several problems persisted: (1) the recordings were negatively affected by poor stability of the implant due to the flexible skulls of the immature rats and resulting movement artifacts (Figure 5.1); (2) the feedback-controlled instrumentation that regulated temperature in the hypoxia treatment chamber created electrical noise that negatively affected the recordings; (3) poor signal-to-noise and signal-to-artifact ratios made quantitative analyses of the signal difficult; (4) wired-system implants were extremely vulnerable to damage by the dam when pups were returned to their cage. In order to obtain EEG signals with

good signal-to-noise ratio, a decision was made to design and develop a proprietary miniature telemetry system for use in rat pups. After a lengthy development and testing period based on an iterative process (Figure 5.2), the miniature telemetry system enabled us to make serial EEG recordings in rat pups as young as P6. This technical development enabled us to quantitatively examine seizures and background patterns in models of neonatal Ha and HIE.

Figure 5.1**: Improvement of the EEG signals recorded from immature rat pups.** Initially, a wired system was used for this project, but signal quality was poor with numerous movement artifacts (A). Technical modifications of the miniature telemetry system led to incremental improvements (B, C) that eventually resulted in high-quality recordings that were virtually artifact-free (D). Artifacts marked in red.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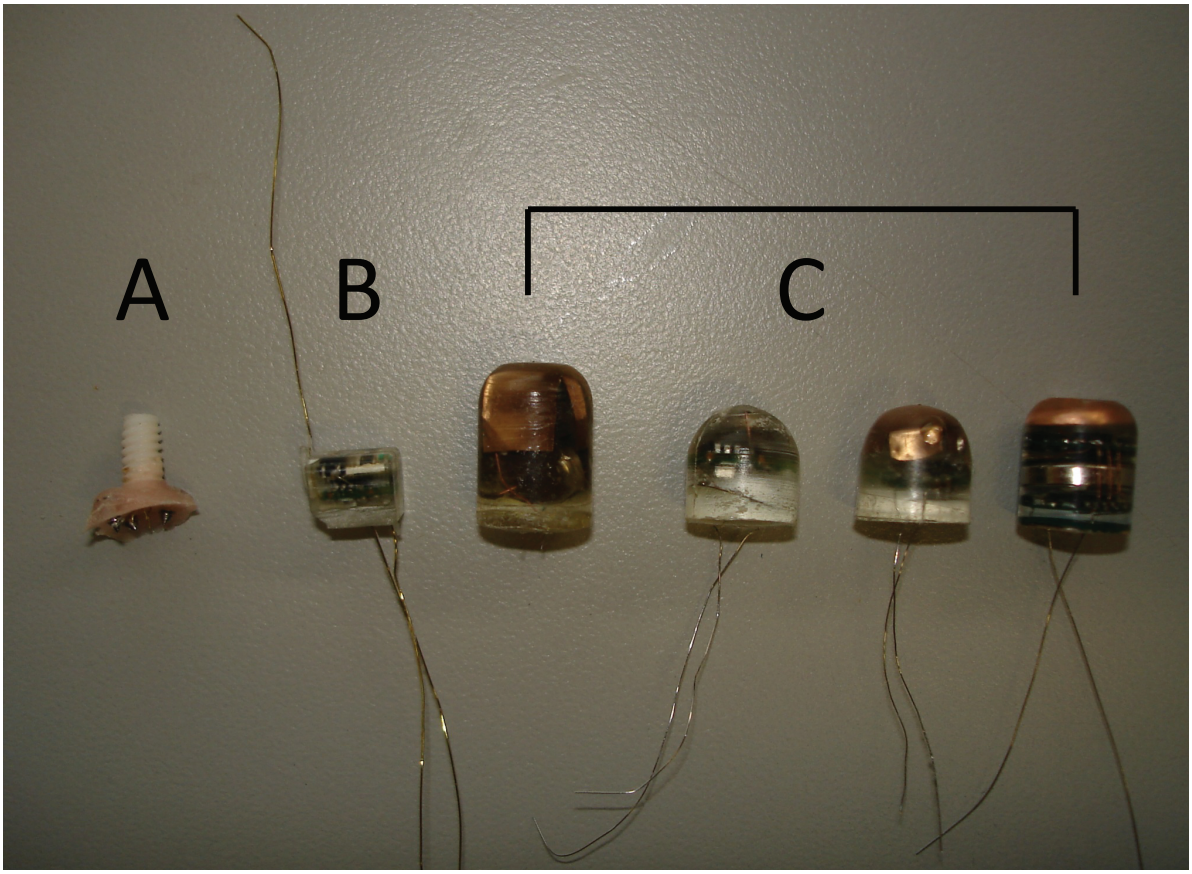

Figure 5.2: **Iterations of the recording systems used in this study.** Plastics One wired electrode (A), photodiode powered telemetry unit in its original form factor (B) and various versions of the miniature wireless telemetry system (C).

Increased intensity of hypoxia-induced seizure activity was not associated with negative outcome

Traditionally, in animal models of neonatal seizures, behavioral seizures have been the only dependent variable analyzed, regardless of the underlying etiology. Clinical studies, however, suggest that the predictive value of seizures is highly dependent on the underlying etiology. In the rat models of hypoxia, seizures caused by Ha have not been reported to result in overt neuronal death (Jensen et al., 1981; Rakhade et al., 2011; Rice et al., 1981; Vannucci and Vannucci., 2009). Our EEG-power analysis shows that Ha-induced seizure activity became *progressively* more *intense* over the 2-h duration of the exposure to Ha. Background activity increased as well, and the animals never appeared to have any EEG features consistent with background suppression. When returned to a normoxic environment, the animals had a slight dysfunction in the alpha band that quickly recovered to the level of the controls. When EEG was quantified using our methods, to assess for both background suppression and presence of subacute seizures, the Ha-treated animals were similar to controls. In this case, the lack of background suppression appeared to be predictive of a positive outcome and *progressively intense* acute seizures were *not* predictive of the negative outcome. While it is possible that in our models, Ha-induced seizures may lead to cognitive abnormalities and lower seizure thresholds later in life (Jensen et al., 1991; Aujla et al., 2009), it seems at this point unlikely that this etiology leads to a profoundly negative outcome. Therefore, when examining

behavioral seizures in these animal models, care must be taken to determine the underlying etiology, which may be identifiable by studying both the ictal *and background* components of the EEG.

A progressive decrease in seizure intensity and development of EEG background suppression was associated with the negative outcome

Quantitative power analysis of seizures in animals with HIE revealed *progressively lower* intensity over time in the HI-treated rats. With this finding, we rejected our initial hypothesis that acute seizures were more severe in animals with neuronal damage (Figure 5.3). The decreased seizure intensity occurred in two frequency bands at two different time points. Vannucci (1990) reported a depletion of phosophocreatine at 20 min in the HI model at P7, which was followed by histologically detectable neuronal death after 90 min of treatment. These time points appear to match the changes in EEG power during HI-induced acute seizures. Our results suggest that in the delta frequency band, the seizure profile was similar in both HI and Ha until about 90 min into the treatment. This decrease in seizure intensity could be interpreted as the underlying dysfunction of the brain (i.e. neuronal death beginning to occur). The seizure activity in the alpha band and the background patterns appeared to decrease in intensity after 20 min of HI administration. This latter change could be interpreted as the first sign of the dysfunction in the brain when high energy reserves become depleted.

Thus, it appears that while seizures in both models can be elicited by hypoxia, it is the background suppression and the reduction in EEG power during seizures that are indicative of underlying catastrophic dysfunction. This finding matches what was previously reported in the clinical literature: seizure activity superimposed on suppressed background is predictive of an extremely negative outcome in as much as 90% of the cases examined (Volpe et al., 2001; Lombroso, 1983; Rowe et al., 1985; Menache et al., 2002). Thus, decreases in cerebral activity in both seizures and background were associated with HIE in this animal model.

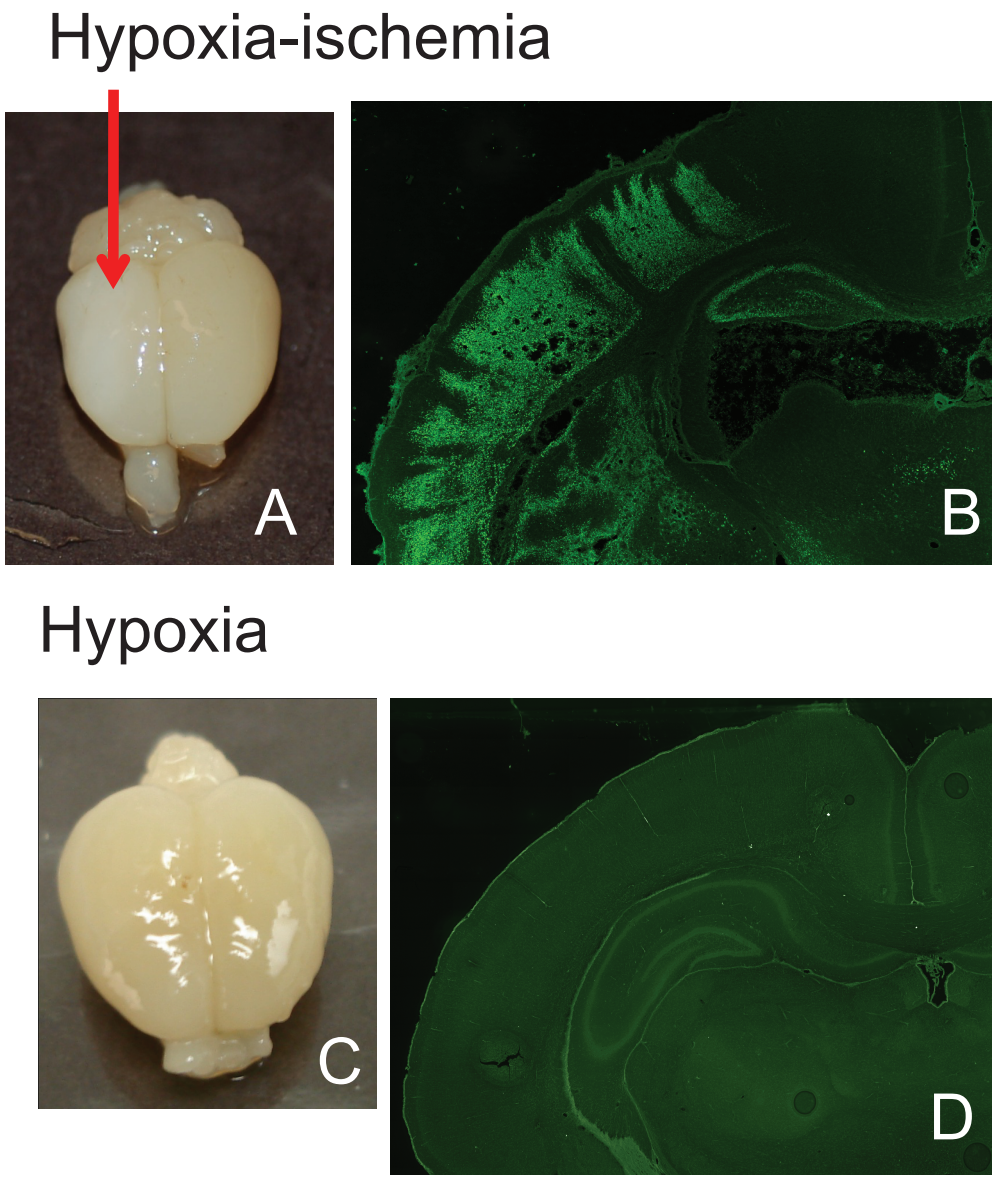

Figure 5.3: **Typical lesion in the HI-treated animal compared to the control brain.** HI-treated animals had a macroscopically identifiable lesion 96 h after the insult. The cells inside the lesion were Flouro-Jade B positive. Ha-treated animals had no lesion or Flouro-Jade B positive cells.

Relationship between acute and subacute seizures

Subacute seizures are an important measure, because they are likely caused by the altered excitability from the dysfunctional network circuits or anatomical abnormality in the brain, as compared to the seizures that are triggered by acute Ha. The acute and subacute seizures had different morphological waveforms (Figure 2.4; Figure 3.6). We were able to connect sub-acute seizures that were recorded in three HI-treated animals with the background suppression typical to HIE. Because electrographic seizures were only detected during the *subacute* period in the HI group, it is highly probable that they appeared in the animals that had progressively reduced *acute* seizure intensity. It is likely that the brain damage in the animals with HIE caused the reduction of acute seizures, the presence of background suppression and the subsequent appearance of subacute seizures. Thus, it appears that acute seizures do not directly cause the subacute seizures; instead, it was the presence of the neuronal lesion that caused subacute seizures. A caveat to this interpretation is the study design, where the acute and subacute experiments were conducted in two different experimental groups. Additionally, it is likely that animals with HIE had more subacute seizures, but that they were missed because the monitoring protocol was intermittent. A continuous (i.e., “24/7”) monitoring protocol for prolonged periods would be required to address this question. Longer monitoring periods increase the likelihood of detecting other abnormalities in both HI and Ha-treated animals. During the time when these

experiments were conducted, continuous 24-h monitoring was not possible. The most recent iterations of the miniature telemetry system will now allow continuous 24/7 monitoring of the naturally reared pups, making future experimental studies to better assess subacute seizures and the subsequent development of epilepsy much more feasible. Thus, it appears that HIE-induced background suppression in the acute and subacute periods was associated with and subsequent negative outcome (i.e., the lesion), and this in turn was associated with the subacute seizures.

Sensitivity of the background monitoring approach

With the HIE model used in these studies, it was difficult to test how the size and the exact location of the lesion affected the EEG signal; the animals either had no lesion, catastrophic lesions or they died. A previous study in our lab by Kadam and colleagues (2010) reported that only 50% of HI-treated animals had lesions. The methods and the instrumentation that were used in their study were slightly different to the ones described here. First, in the Kadam et al. (2010) study, the carotid artery in the pups was occluded with two sutures, but was not cut; in the present study the carotid artery was cut by cauterization. In those cases where the arteries were occluded with sutures, eventual reperfusion is possible, while this possibility is completely eliminated by cutting and cauterizing the artery. Second, the design of the instrumentation used to administer HI was different in the two studies. In the Kadam study, a retrofitted

egg incubator was used for administration of hypoxia. The incubator did not utilize feedback for controlling the temperature, thus it is possible that the temperature inside of the incubator varied slightly due to variations in the ambient room temperature. Our design used a treatment chamber where temperature was regulated with a proportional-integral-derivative (PID) controller by using feedback via a thermocouple placed close to the chamber housing the animal. Temperature variations and reperfusion were both previously reported to affect the size of the resulting infarct in stroke models. One possible outlier (n=1) was present in our study. In this animal, the HI-induced lesion was absent in the *cortex*, but still present in the hippocampus and thalamus (Figure 5.4). Despite the absence of the cortical lesion, the animal still had suppressed background in both early and late recording groups (Figure 5.4 A-F). The suppression, however, was not as severe as in the animals with cortical lesions. Thus, it is plausible that background abnormalities are not just a measure of cortical dysfunction, but could occur due to lesions deeper in the brain. Future studies in models where location and size of the lesion can be controlled more effectively would be needed to further explore this finding.

Acute and subacute findings:

translational value

EEG recordings *during* the administration of the insult may initially seem to have limited translational value, because in clinical practice the detection,

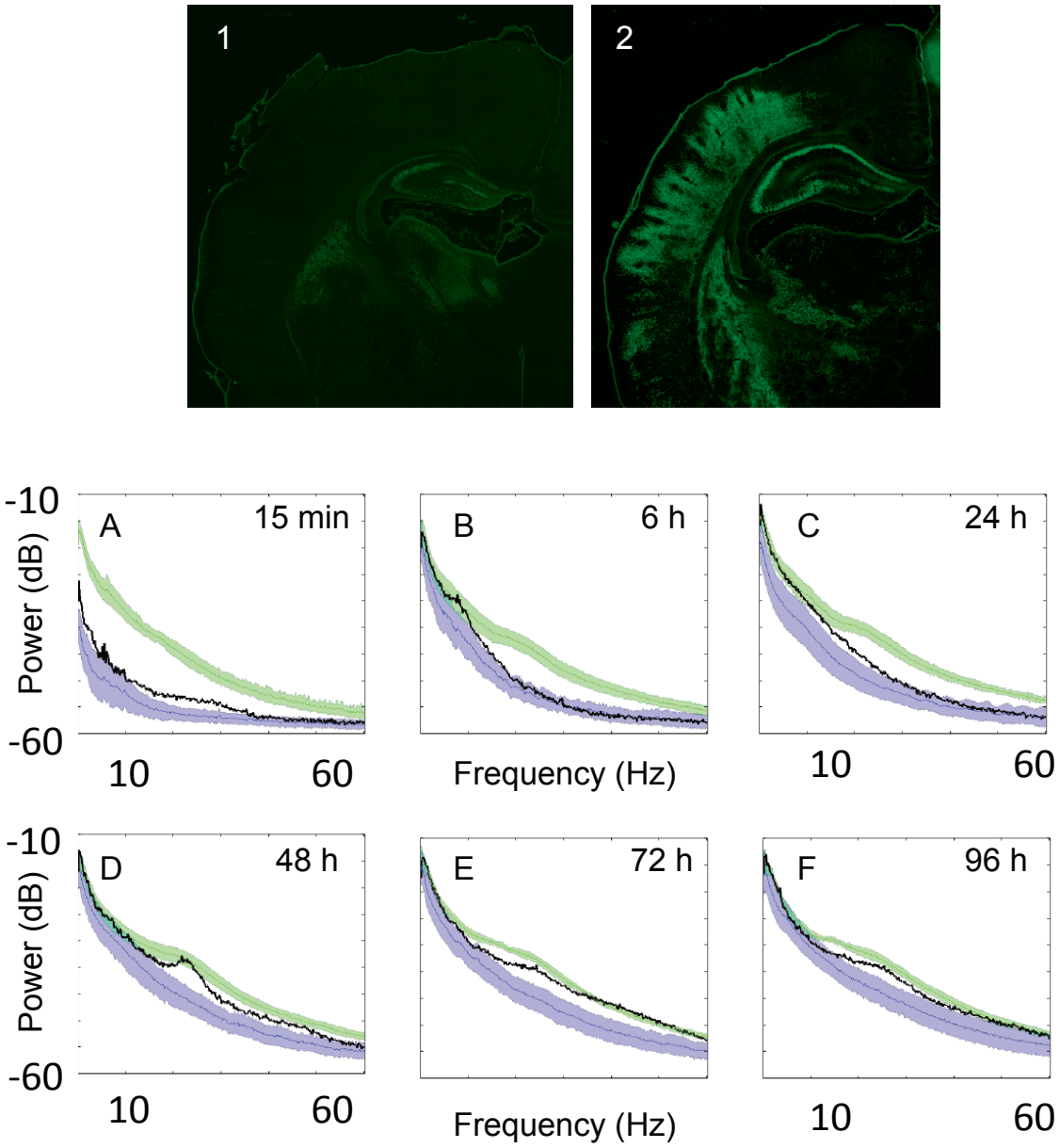

Figure 5.4: Animal with an incomplete lesion and the underlying signal patterns. The following animal has lesion in hippocampus and thalamus as detected by Flouro-Jade B (1). The lesion was missing the typical cortical component found in most of the animals (2). Data was plotted using mean PSD with 95% confidence intervals from all HI animals without the outlier (blue), all control animals (green) and the outlier animal with an atypical lesion (black). Despite the absence of the cortical lesion (i.e., only the subcortical lesion was present), background suppression was observed in the EEG signal (A-F).

management and interventions are likely to occur during the subacute period. Most neonatal insults are thought to occur during labor and delivery, a period when cerebral monitoring is much more difficult. However, there is evidence that intrapartum difficulties do not always *directly* cause an insult, instead they may increase the probability of new insults in the postnatal period (Cowan et al., 2003). In this case, animal models could provide us with data enabling early detection and intervention for HIE and stroke. The drawback of this approach is that continuous monitoring of cerebral activity in all at-risk individuals would be required from the moment of delivery. In the subacute period, the power and frequency analysis of the background EEG yielded the most promising quantifiable measure that was predictive of outcome. The EEG patterns could be used not only as predictors of whether HIE was present, but could also be used to determine the time after the insult when brain injury started to occur. This concept has potential to be useful for screening neonates that would benefit most from hypothermic intervention. Hypothermia has been described to be most beneficial when administered within 6 h after the insult; however, due to prolonged or delayed clinical evaluations, this therapeutic window is often missed (Khurshid et al., 2011; Thoresen et al., 1995; Gunn et al., 1998; Azzorardi et al., 2009). In the rodent model of HIE used here, the background activity pattern in the *early* 15-min group was significantly different from that in the *late* 6-h group. In the early recordings after the insult, the background activity showed suppression in both the low- and high frequency bands. After 6 h, the low-frequency activity recovered, while high frequency patterns remained

suppressed. Thus, it is possible - and would need to be examined experimentally that the low frequency background suppression in the early group is an indicator of the time point when further brain injury would be most preventable. The *early* suppression of EEG power in low-frequency bands is a potential screening measure for early application of hypothermia. Both acute and subacute findings appear to have translational value for different types of insults – acute monitoring for detection of ongoing perinatal insults and subacute screening for best candidates for hypothermia therapy.

Early detection of neurologic abnormalities with minimal monitoring

Abnormalities such as epileptiform discharges and seizures are generally periodic or clustered. Monitoring over prolonged periods of time is often necessary to detect and quantify them in an accurate manner. Because background suppression is a constant and persistent change in the EEG signal, much shorter recording periods can be used to detect this abnormality. Korotchikova and colleagues (2011) provided a proof-of-concept for the use of quantitative background EEG in humans for determining the grade of HIE *retrospectively*. The human EEG traces were qualitatively similar to those shown in this study using rodent models (Figure 5.5). In the animal model, our results suggest that in the early groups, background suppression could be detected using recording epochs of 1-min duration. The concept of using such short recording epochs would have to be independently validated using human data,

which might be difficult because early detection of the insult is problematic in humans in the first place (Volpe et al., 2001; Nelson and Lynch, 2004). Previously, cerebral activity monitors, such as amplitude-integrated EEG (aEEG), have required lengthy recording epochs and have primarily been used to monitor the progress of the patient during administration of the therapy. Instead, we propose the concept of a device that could be developed for screening of all neonates at risk. Conceptually, such a device would have low specificity for the cause of dysfunction, but high sensitivity for ascertaining abnormalities, such as background suppression. While this approach would not provide the user with a specific underlying cause, it could enable screening of neonates at any time point during their stay in the hospital after delivery for possible abnormalities without the use of invasive instrumentation. Such an approach would enable *prospective* screening for potentially negative and catastrophic etiologies.

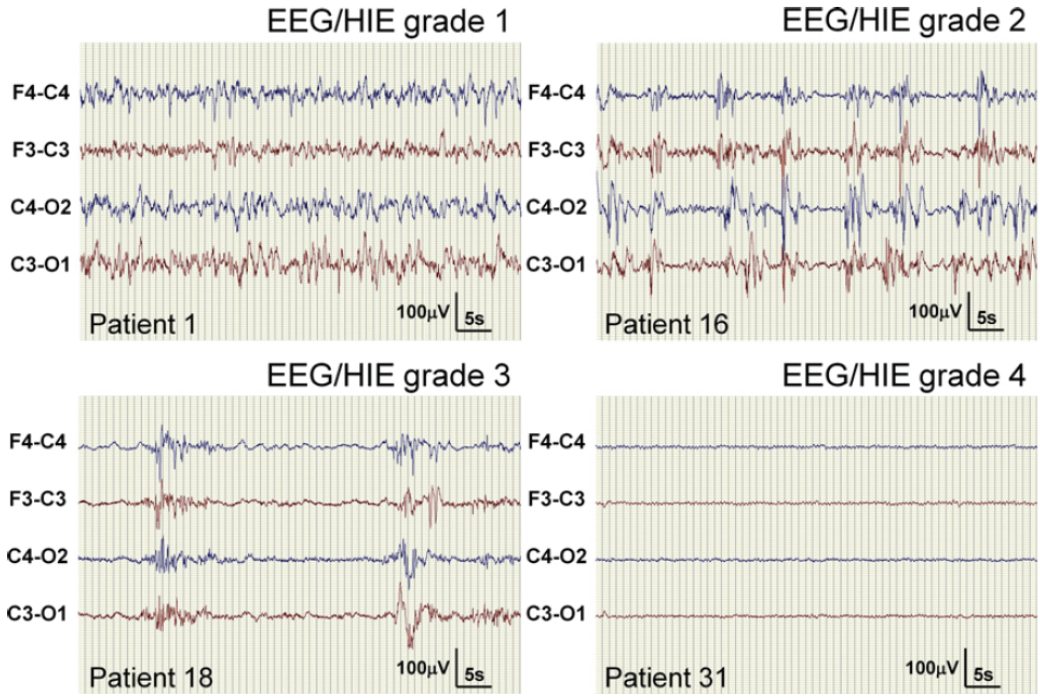

Figure 5.5: **Examples of graded EEG recordings from human neonates with hypoxic-ischemic encephalopathy.** Taken from Korotchikova et al., (2011); *Clinical Neurophys.,* 122 (8), p1671-8

Future Directions

Dynamic monitoring and interventions in the animal models

Obtaining real time data from neonatal animals allows for dynamic monitoring of physiological parameters in the models of perinatal insults. In humans, the progression of HIE is not linear; a specific number of changes occur during the first 72 h of the insult. For example, Volpe (2001) described in neonates with HIE that specific changes occur as a function of time (0-12 h; 12-24 h; 24-72 h) after insult. In the HI model, we detected specific abnormalities that occur and progressively evolve during and after the insult. Currently, it is common in the animal models to use pretreatment protocols for testing of new potential therapies (Raol et al., 2009; Dzhala et al., 2005; Aujla et al., 2009; Koh and Jensen, 2001). Such an approach would probably only affect the initiation of the insult, and could possibly confound the progressive evolution of the insult, resulting in a false positive or a false negative outcome. Telemetric recordings have the potential to test single therapies or combinations of therapies at various points of the insult while monitoring the progression in real time. This approach would also help identify *specific* time points to be targeted with a therapy and the effect of the therapies at each of these time points. Such an approach has the potential to improve preclinical drug development and testing.

Hypothermia

Hypothermia is becoming a standard-of-care for neonatal HIE; therefore, it is the most logical intervention that we can test in our model. While our results

lead to speculation that the early time period after HI would be the most beneficial for application of therapy, the hypothesis was not experimentally tested. Our current treatment/recording chamber is designed to maintain the temperature at a set threshold; however, due to a large volume of circulating heated air in the chamber, a rapid change of temperature is not possible. A new design that would make these experiments possible is currently under development. The newly designed chamber will include a feedback-controlled cartridge heater placed inside of a ceramic tube in series with the manifold on the gas tube. The heating unit will be housed inside of a machined graphite block for heat resistance. The cold air pressure from the tank will be fed through a ceramic tube with the cartridge heater and heated to the required temperature. The threshold for lower temperature will be modulated by the inherently cool air or hypoxia mixture in the tank. Combining hypothermia treatment with the EEG recordings will enable us to detect not only EEG features associated with damage, but also identify features associated with recovery.

Modulating the size of the lesion

The underlying feature of the HI model was that the outcome was catastrophic – animals had macroscopic lesions or died. Purposely modulating the size of the lesion in this model was difficult. Thus, this approach likely simulated clinical worst case scenarios. While one might argue that these individuals would benefit from the intervention the most, we must develop our concept of EEG analysis in more intermediate phenotypes. To accomplish this,

models such as middle cerebral artery occlusion and endothelin-induced lesions could be utilized. Using these approaches, we can further the link between specific electrographic abnormalities and the underlying etiologies.

Twenty-four hour continuous monitoring

Recordings in the subacute period of the injury were performed using an intermittent recording protocol with 2-h recording periods. While this approach provided valuable data on background abnormalities, the results that report sub-acute seizures were likely inconclusive. In order to comprehensively examine subacute seizures, continuous “24/7” monitoring periods must be employed. Until recently, the pups had to be returned to the dam to ensure their survival. We have now developed the newest iteration of the miniature telemetry system that allows performing recordings from the pups that are housed in the cage with their dam and littermates. This approach will enable future studies to collect continuous recordings and further classify subacute seizures and examine their role in HIE.

Translation to human brain injuries

All of the work in this thesis has been done on rat models, although it has also been possible to record from both mice and pre-term lambs during brain insults. Furthermore, the concepts, instrumentation and analytical tools developed here should be directly applicable to human neonates. Our group is now developing these strategies for use in humans with the goal of rapidly

detecting HIE in a manner that would quickly identify those most in need and most likely to benefit from the newest and most sophisticated therapeutic approaches, such as hypothermia.

REFERENCES

Abend NS, Gutierrez-Colina AM, Topjian AA, Zhao H, Guo R, Donnelly M, Clancy RR, Dlugos DJ (2011) Nonconvulsive seizures are common in critically ill children+. Neurology 76:1071-1077.

Abend NS, Gutierrez-Colina A, Zhao H, Guo R, Marsh E, Clancy RR, Dlugos DJ (2011) Interobserver reproducibility of electroencephalogram interpretation in critically ill children. J Clin Neurophysiol 28:15-19.

Abend NS, Topjian AA, Gutierrez-Colina AM, Donnelly M, Clancy RR, Dlugos DJ (2011) Impact of continuous EEG monitoring on clinical management in critically ill children. Neurocrit Care 15:70-75.

Aneja S, Ahuja B, Taluja V, Bhatia VK (2001) Epilepsy in children with cerebral palsy. Indian J Pediatr 68:111-115.

Aso K, Scher MS, Barmada MA (1990) Cerebral infarcts and seizures in the neonate. J Child Neurol 5:224-228.

Aujla PK, Fetell MR, Jensen FE (2009) Talampanel suppresses the acute and chronic effects of seizures in a rodent neonatal seizure model. Epilepsia 50:694-701.

Azzopardi DV, Strohm B, Edwards AD, Dyet L, Halliday HL, Juszczak E,

Kapellou O, Levene M, Marlow N, Porter E, Thoresen M, Whitelaw A, Brocklehurst P (2009) Moderate hypothermia to treat perinatal asphyxial encephalopathy. N Engl J Med 361:1349-1358.

Baram, T. Z., Jensen, F. E., & Brooks-Kayal, A. (2011). Does acquired epileptogenesis in the immature brain require neuronal death. *Epilepsy currents / American Epilepsy Society*, *11*(1), 21-6.

Berg, A. T., & Shinnar, S. (1991). The risk of seizure recurrence following a first unprovoked seizure: a quantitative review. Neurology, 41(7), 965-72.

Bergamasco B, Benna P, Ferrero P, Gavinelli R (1984) Neonatal hypoxia and epileptic risk: a clinical prospective study. Epilepsia 25:131-136.

Bhardwaj SK, Forcelli PA, Palchik G, Gale K, Srivastava LK, Kondratyev A (2012) Neonatal exposure to phenobarbital potentiates schizophrenia-like behavioral outcomes in the rat. Neuropharmacology 62:2337-2345.

Biran V, Heine VM, Verney C, Sheldon RA, Spadafora R, Vexler ZS, Rowitch DH, Ferriero DM (2011) Cerebellar abnormalities following hypoxia alone compared to hypoxic-ischemic forebrain injury in the developing rat brain. Neurobiol Dis 41:138-146.

Bjorkman ST, Miller SM, Rose SE, Burke C, Colditz PB (2010) Seizures are associated with brain injury severity in a neonatal model of hypoxia-ischemia. Neuroscience 166:157-167.

Bourgeois BF, Prensky AL, Palkes HS, Talent BK, Busch SG (1983) Intelligence in epilepsy: a prospective study in children. Ann Neurol 14:438-444.

Boylan GB, Rennie JM, Pressler RM, Wilson G, Morton M, Binnie CD (2002) Phenobarbitone, neonatal seizures, and video-EEG. Arch Dis Child Fetal Neonatal Ed 86:F165-F170.

Camfield PR (1997) Recurrent seizures in the developing brain are not harmful. Epilepsia 38:735-737.

CDC, (2004), Economic costs associated with mental retardation, cerebral palsy, hearing loss, and vision impairment--United States, 2003. *MMWR. Morbidity and mortality weekly report*, *53*(3), 57-9.

Chabwine JN, Vanden Eijnden S (2011) A claim for caution in the use of promising bumetanide to treat neonatal seizures. J Child Neurol 26:657-658.

Chen J, Cai F, Cao J, Zhang X, Li S (2009) Long-term antiepileptic drug administration during early life inhibits hippocampal neurogenesis in the developing brain. J Neurosci Res 87:2898-2907.

Clancy RR, Legido A, Lewis D (1988) Occult neonatal seizures. Epilepsia 29:256-261.

Clancy RR, Legido A (1991) Postnatal epilepsy after EEG-confirmed neonatal seizures. Epilepsia 32:69-76.

Connell J, Oozeer R, de VL, Dubowitz LM, Dubowitz V (1989) Clinical and EEG response to anticonvulsants in neonatal seizures. Arch Dis Child 64:459-464.

Cowan F, Rutherford M, Groenendaal F, Eken P, Mercuri E, Bydder GM, Meiners LC, Dubowitz LM, de Vries LS (2003) Origin and timing of brain lesions in term infants with neonatal encephalopathy. Lancet 361:736-742.

Corey, L., Whitley, R. J., Stone, E. F., & Mohan, K. (1988). Difference between herpes simplex virus type 1 and type 2 neonatal encephalitis in neurological outcome. *Lancet*, *1*(8575-6), 1-4.

Cuaycong M, Engel M, Weinstein SL, Salmon E, Perlman JM, Sunderam S, Vannucci SJ (2011) A novel approach to the study of hypoxia-ischemia-induced clinical and subclinical seizures in the neonatal rat. Dev Neurosci 33:241-250.

Cummins SK, Nelson KB, Grether JK, Velie EM (1993) Cerebral palsy in four northern California counties, births 1983 through 1985. J Pediatr 123:230-237.

Dohmen C, Sakowitz OW, Fabricius M, Bosche B, Reithmeier T, Ernestus RI, Brinker G, Dreier JP, Woitzik J, Strong AJ, Graf R (2008) Spreading depolarizations occur in human ischemic stroke with high incidence. Ann Neurol 63:720-728.

Dreier JP, Major S, Manning A, Woitzik J, Drenckhahn C, Steinbrink J, Tolias C, Oliveira-Ferreira AI, Fabricius M, Hartings JA, Vajkoczy P, Lauritzen M, Dirnagl U, Bohner G, Strong AJ (2009) Cortical spreading ischaemia is a novel process involved in ischaemic damage in patients with aneurysmal subarachnoid haemorrhage. Brain 132:1866-1881.

Drenckhahn C, Winkler MK, Major S, Scheel M, Kang EJ, Pinczolits A, Grozea C, Hartings JA, Woitzik J, Dreier JP (2012) Correlates of spreading depolarization in human scalp electroencephalography. Brain 135:853-868.

du Plessis AJ, Volpe JJ (2002) Perinatal brain injury in the preterm and term newborn. Curr Opin Neurol 15:151-157.

Dube C, Chen K, Eghbal-Ahmadi M, Brunson K, Soltesz I, Baram TZ (2000) Prolonged febrile seizures in the immature rat model enhance hippocampal excitability long term. Ann Neurol 47:336-344.

Dube CM, Ravizza T, Hamamura M, Zha Q, Keebaugh A, Fok K, Andres AL, Nalcioglu O, Obenaus A, Vezzani A, Baram TZ (2010) Epileptogenesis provoked by prolonged experimental febrile seizures: mechanisms and biomarkers. J Neurosci 30:7484-7494.

Baram, T. Z., Jensen, F. E., & Brooks-Kayal, A. (2011). Does acquired epileptogenesis in the immature brain require neuronal death. *Epilepsy currents / American Epilepsy Society*, *11*(1), 21-6.

Dzhala VI, Talos DM, Sdrulla DA, Brumback AC, Mathews GC, Benke TA, Delpire E, Jensen FE, Staley KJ (2005) NKCC1 transporter facilitates seizures in the developing brain. Nat Med 11:1205-1213.

Ekstrand JJ, Pouliot W, Scheerlinck P, Dudek FE (2011) Lithium pilocarpine-induced status epilepticus in postnatal day 20 rats results in greater neuronal injury in ventral versus dorsal hippocampus. Neuroscience 192:699-707.

Finer, N. N., Robertson, C. M., Richards, R. T., Pinnell, L. E., & Peters, K. L. (1981). Hypoxic-ischemic encephalopathy in term neonates: perinatal factors and outcome. The Journal of pediatrics, *98*(1), 112-7

Fitzgerald KC, Williams LS, Garg BP, Golomb MR (2007) Epilepsy in children with delayed presentation of perinatal stroke. J Child Neurol 22:1274-1280.

Folbergrova J (1994) NMDA and not non-NMDA receptor antagonists are protective against seizures induced by homocysteine in neonatal rats. Exp Neurol 130:344-350.

Forcelli PA, Kim J, Kondratyev A, Gale K (2011) Pattern of antiepileptic drug-induced cell death in limbic regions of the neonatal rat brain. Epilepsia 52:e207-e211.

Forcelli PA, Janssen MJ, Vicini S, Gale K (2012) Neonatal exposure to antiepileptic drugs disrupts striatal synaptic development. Ann Neurol.

Friedman, L. K., Pellegrini-Giampietro, D. E., Sperber, E. F., Bennett, M. V., Moshé, S. L., & Zukin, R. S. (1994). Kainate-induced status epilepticus alters glutamate and GABAA receptor gene expression in adult rat hippocampus: an in situ hybridization study. J. Neurosci, *14*(5 Pt 1), 2697-707

Friedman, L. K., Sperber, E. F., Moshé, S. L., Bennett, M. V., & Zukin, R. S. (1997). Developmental regulation of glutamate and GABA(A) receptor gene expression in rat hippocampus following kainate-induced status epilepticus. Developmental neuroscience, 19(6), 529-42.

Gal P, Toback J, Erkan NV (1984) The influence of asphyxia on phenobarbital dosing requirements in neonates . Dev. Pharmacol Ther 7:145-152

Garfinkle J, Shevell MI (2011) Predictors of outcome in term infants with neonatal seizures subsequent to intrapartum asphyxia. J Child Neurol 26:453-459.

Germano, I. M., Zhang, Y. F., Sperber, E. F., & Moshé, S. L. (1996). Neuronal migration disorders increase susceptibility to hyperthermia-induced seizures in developing rats. *Epilepsia*, *37*(9), 902-10

Germano, I. M., Sperber, E. F., Ahuja, S., & Moshé, S. L. (1998). Evidence of enhanced kindling and hippocampal neuronal injury in immature rats with neuronal migration disorders. *Epilepsia*, *39*(12), 1253-60.

Gilman, J. T., Gal, P., Duchowny, M. S., Weaver, R. L., & Ransom, J. L. (1989). Rapid sequential phenobarbital treatment of neonatal seizures. *Pediatrics*, *83*(5), 674-8.

Glass HC, Glidden D, Jeremy RJ, Barkovich AJ, Ferriero DM, Miller SP (2009) Clinical Neonatal Seizures are Independently Associated with Outcome in Infants at Risk for Hypoxic-Ischemic Brain Injury. J Pediatr 155:318-323.

Glass HC, Wirrell E (2009) Controversies in neonatal seizure management. J Child Neurol 24:591-599.

Gloor, P., Ball, G., & Schaul, N. (1977). Brain lesions that produce delta waves in the EEG. *Neurology*, *27*(4), 326-33.

Glykys J, Dzhala VI, Kuchibhotla KV, Feng G, Kuner T, Augustine G, Bacskai BJ, Staley KJ (2009) Differences in cortical versus subcortical GABAergic signaling: a candidate mechanism of electroclinical uncoupling of neonatal seizures. Neuron 63:657-672.

Gunn AJ, Gunn TR, Gunning MI, Williams CE, Gluckman PD (1998) Neuroprotection with prolonged head cooling started before postischemic seizures in fetal sheep. Pediatrics 102:1098-1106.

Hasson H, Malhotra S, Giorgi FS, Rosenbaum DM, Moshe SL (2010) Harmful effect of kainic acid on brain ischemic damage is not related to duration of status epilepticus. Neurol Sci 31:103-105.

Hellstrom-Westas L, Rosen I (2005) Electroencephalography and brain damage in preterm infants. Early Hum Dev 81:255-261.

Hill A, Volpe JJ (1982) Hypoxic-ischemic brain injury in the newborn. Semin Perinatol 6:25-41.

Hirsch E, Baram TZ, Snead OC, III (1992) Ontogenic study of lithium-pilocarpine-induced status epilepticus in rats. Brain Res 583:120-126.

Holmes GL, Lombroso CT (1993) Prognostic value of background patterns in the neonatal EEG. J Clin Neurophysiol 10:323-352.

Holtmaat AJ, Gorter JA, De WJ, Tolner EA, Spijker S, Giger RJ, Lopes da Silva FH, Verhaagen J (2003) Transient downregulation of Sema3A mRNA in a rat model for temporal lobe epilepsy. A novel molecular event potentially contributing to mossy fiber sprouting. Exp Neurol 182:142-150.

Hossain, M. A. (2005). Molecular mediators of hypoxic-ischemic injury and implications for epilepsy in the developing brain. Epilepsy & behavior : E&B, *7*(2), 204-13.

Jensen, F. E., Applegate, C. D., Holtzman, D., Belin, T. R., & Burchfiel, J. L. (1991). Epileptogenic effect of hypoxia in the immature rodent brain. Annals of neurology, *29*(6), 629-37.

Jensen FE, Blume H, Alvarado S, Firkusny I, Geary C (1995) NBQX blocks acute and late epileptogenic effects of perinatal hypoxia. Epilepsia 36:966-972.

Jensen FE, Wang C, Stafstrom CE, Liu Z, Geary C, Stevens MC (1998) Acute and chronic increases in excitability in rat hippocampal slices after perinatal hypoxia In vivo. J Neurophysiol 79:73-81.

Kadam SD, Dudek FE (2007) Neuropathogical features of a rat model for perinatal hypoxic-ischemic encephalopathy with associated epilepsy. J Comp Neurol 505:716-737.

Kadam SD, White AM, Staley KJ, Dudek FE (2010) Continuous electroencephalographic monitoring with radio-telemetry in a rat model of perinatal hypoxia-ischemia reveals progressive post-stroke epilepsy. J Neurosci 30:404-415.

Khazipov R, Sirota A, Leinekugel X, Holmes GL, Ben-Ari Y, Buzsaki G (2004) Early motor activity drives spindle bursts in the developing somatosensory cortex. Nature 432:758-761.

Khurshid F, Lee KS, McNamara PJ, Whyte H, Mak W (2011) Lessons learned during implementation of therapeutic hypothermia for neonatal hypoxic ischemic encephalopathy in a regional transport program in Ontario. Paediatr Child Health 16:153-156.

Kilb, W., Sinning, A., & Luhmann, H. J. (2007). Model-specific effects of bumetanide on epileptiform activity in the in-vitro intact hippocampus of the newborn mouse. Neuropharmacology, *53*(4), 524-33.

Koh S, Jensen FE (2001) Topiramate blocks perinatal hypoxia-induced seizures in rat pups. Ann Neurol 50:366-372.

Koh S, Tibayan FD, Simpson JN, Jensen FE (2004) NBQX or topiramate treatment after perinatal hypoxia-induced seizures prevents later increases in seizure-induced neuronal injury. Epilepsia 45:569-575.

Kondo S, Najm I, Kunieda T, Perryman S, Yacubova K, Luders HO (2001) Electroencephalographic characterization of an adult rat model of radiation-induced cortical dysplasia. Epilepsia 42:1221-1227.

Korotchikova I, Connolly S, Ryan CA, Murray DM, Temko A, Greene BR, Boylan GB (2009) EEG in the healthy term newborn within 12 hours of birth. Clin Neurophysiol 120:1046-1053.

Korotchikova I, Stevenson NJ, Walsh BH, Murray DM, Boylan GB (2011) Quantitative EEG analysis in neonatal hypoxic ischaemic encephalopathy. Clin Neurophysiol 122:1671-1678.

Lai MC, Lui CC, Yang SN, Wang JY, Huang LT (2009) Epileptogenesis is increased in rats with neonatal isolation and early-life seizure and ameliorated by MK-801: a long-term MRI and histological study. Pediatr Res 66:441-447.

Lamblin MD, Andre M, Challamel MJ, Curzi-Dascalova L, d'Allest AM, De Giovanni E, Moussalli-Salefranque F, Navelet Y, Plouin P, Radvanyi-Bouvet MF, Samson-Dollfus D, Vecchierini-Blineau MF (1999) [Electroencephalography of the premature and term newborn. Maturational aspects and glossary]. Neurophysiol Clin 29:123-219.

Lanska MJ, Lanska DJ, Baumann RJ, Kryscio RJ (1995) A population-based study of neonatal seizures in Fayette County, Kentucky. Neurology 45:724-732.

Lanska MJ, Lanska DJ (1996) Neonatal seizures in the United States: results of the National Hospital Discharge Survey, 1980-1991. Neuroepidemiology 15:117-125.

Lawn JE, Cousens S, Zupan J (2005) 4 million neonatal deaths: when? Where? Why? Lancet 365:891-900.

Lee J, Croen LA, Lindan C, Nash KB, Yoshida CK, Ferriero DM, Barkovich AJ, Wu YW (2005) Predictors of outcome in perinatal arterial stroke: a population-based study. Ann Neurol 58:303-308.

Legido A, Clancy RR, Berman PH (1991) Neurologic outcome after electroencephalographically proven neonatal seizures. Pediatrics 88:583-596.

Lehmkuhle MJ, Thomson KE, Scheerlinck P, Pouliot W, Greger B, Dudek FE (2009) A simple quantitative method for analyzing electrographic status epilepticus in rats. J Neurophysiol 101:1660-1670.

Levene MI (1993) Management of the asphyxiated full term infant. Arch Dis Child 68:612-616.

Levine, S. (1960). Anoxic-ischemic encephalopathy in rats. The American journal of pathology, 36, 1-17.

Lombroso CT (1983) Prognosis in neonatal seizures. Adv Neurol 34:101-113.

Lynch JK, Hirtz DG, DeVeber G, Nelson KB (2002) Report of the National Institute of Neurological Disorders and Stroke workshop on perinatal and childhood stroke. Pediatrics 109:116-123.

MacDonald HM, Mulligan JC, Allen AC, Taylor PM. (1980) Neonatal asphyxia. I. Relationship of obstetric and neonatal complications to neonatal mortality in 38,405 consecutive deliveries. Journal of Pediatrics 95 (5), 898-902

Malm, G. (2009). Neonatal herpes simplex virus infection. Seminars in fetal & neonatal medicine, 14(4), 204-8.

Marin-Padilla M (2000) Perinatal brain damage, cortical reorganization (acquired cortical dysplasias), and epilepsy. Adv Neurol 84:153-172.

Maytal J, Shinnar S, Moshe SL, Alvarez LA (1989) Low morbidity and mortality of status epilepticus in children. Pediatrics 83:323-331.

Menache CC, Bourgeois BF, Volpe JJ (2002) Prognostic value of neonatal discontinuous EEG. Pediatr Neurol 27:93-101.

Mercuri E, Cowan F, Rutherford M, Acolet D, Pennock J, Dubowitz L (1995) Ischaemic and haemorrhagic brain lesions in newborns with seizures and normal Apgar scores. Arch Dis Child Fetal Neonatal Ed 73:F67-F74.

Mercuri E, Rutherford M, Cowan F, Pennock J, Counsell S, Papadimitriou M, Azzopardi D, Bydder G, Dubowitz L (1999) Early prognostic indicators of outcome in infants with neonatal cerebral infarction: a clinical, electroencephalogram, and magnetic resonance imaging study. Pediatrics 103:39-46.

Mies, G., Iijima, T., & Hossmann, K. A. (1993). Correlation between peri-infarct DC shifts and ischaemic neuronal damage in rat. *Neuroreport*, *4*(6), 709-11

Mikati MA, El Hokayem JA, El Sabban ME (2007) Effects of a single dose of erythropoietin on subsequent seizure susceptibility in rats exposed to acute hypoxia at P10. Epilepsia 48:175-181.

Mizrahi EM, Kellaway P (1984) Cerebral concussion in children: assessment of injury by electroencephalography. Pediatrics 73:419-425.

Mizrahi EM (1987) Neonatal seizures: problems in diagnosis and classification. Epilepsia 28 Suppl 1:S46-S55.

Mizrahi EM (1998) Alternate endpoint: EEG assessment of antiepileptic drug efficacy and toxicity. Adv Neurol 76:209-221.

Mizrahi EM, Clancy RR (2000) Neonatal seizures: early-onset seizure syndromes and their consequences for development. Ment Retard Dev Disabil Res Rev 6:229-241.

Monod N, Pajot N, Guidasci S (1972) The neonatal EEG: statistical studies and prognostic value in full-term and pre-term babies. Electroencephalogr Clin Neurophysiol 32:529-544.

Moshe SL (1998) Brain injury with prolonged seizures in children and adults. J Child Neurol 13 Suppl 1:S3-S6.

Murray DM, Boylan GB, Ryan CA, Connolly S (2009) Early EEG findings in hypoxic-ischemic encephalopathy predict outcomes at 2 years. Pediatrics 124:e459-e467.

Murray DM, Bala P, O'Connor CM, Ryan CA, Connolly S, Boylan GB (2010) The predictive value of early neurological examination in neonatal hypoxic-ischaemic encephalopathy and neurodevelopmental outcome at 24 months. Dev Med Child Neurol 52:e55-e59.

Nash KB, Bonifacio SL, Glass HC, Sullivan JE, Barkovich AJ, Ferriero DM, Cilio MR (2011) Video-EEG monitoring in newborns with hypoxic-ischemic encephalopathy treated with hypothermia. Neurology 76:556-562.

Nelson KB, Ellenberg JH (1976) Predictors of epilepsy in children who have experienced febrile seizures. N Engl J Med 295:1029-1033.

Nelson KB, Lynch JK (2004) Stroke in newborn infants. Lancet Neurol 3:150-158.

Niemarkt HJ, Jennekens W, Pasman JW, Katgert T, Van Pul C, Gavilanes AW, Kramer BW, Zimmermann LJ, Bambang OS, Andriessen P (2011) Maturational changes in automated EEG spectral power analysis in preterm infants. Pediatr Res 70:529-534.

Nitecka L, Tremblay E, Charton G, Bouillot JP, Berger ML, Ben-Ari Y (1984) Maturation of kainic acid seizure-brain damage syndrome in the rat. II. Histopathological sequelae. Neuroscience 13:1073-1094.

Painter MJ, Scher MS, Stein AD, Armatti S, Wang Z, Gardiner JC, Paneth N, Minnigh B, Alvin J (1999) Phenobarbital compared with phenytoin for the treatment of neonatal seizures. N Engl J Med 341:485-489.

Patel J, Edwards AD (1997) Prediction of outcome after perinatal asphyxia. Curr Opin Pediatr 9:128-132.

Pezzani C, Radvanyi-Bouvet MF, Relier JP, Monod N (1986) Neonatal electroencephalography during the first twenty-four hours of life in full-term newborn infants. Neuropediatrics 17:11-18.

Quinn R (2005) Comparing rat's to human's age: how old is my rat in people years? Nutrition 21:775-777.

Raju TN, Nelson KB, Ferriero D, Lynch JK (2007) Ischemic perinatal stroke: summary of a workshop sponsored by the National Institute of Child Health and Human Development and the National Institute of Neurological Disorders and Stroke. Pediatrics 120:609-616.

Rakhade SN, Klein PM, Huynh T, Hilario-Gomez C, Kosaras B, Rotenberg A, Jensen FE (2011) Development of later life spontaneous seizures in a rodent model of hypoxia-induced neonatal seizures. Epilepsia 52:753-765.

Raol, Y. S. H., Budreck, E. C., & Brooks-Kayal, A. R. (2003). Epilepsy after early-life seizures can be independent of hippocampal injury. Annals of neurology, *53*(4), 503-11.

Raol YH, Lapides DA, Keating JG, Brooks-Kayal AR, Cooper EC (2009) A KCNQ channel opener for experimental neonatal seizures and status epilepticus. Ann Neurol 65:326-336.

Rheims S, Minlebaev M, Ivanov A, Represa A, Khazipov R, Holmes GL, Ben Ari Y, Zilberter Y (2008) Excitatory GABA in rodent developing neocortex in vitro. J Neurophysiol 100:609-619.

Rice, J. E., Vannucci, R. C., & Brierley, J. B. (1981). The influence of immaturity on hypoxic-ischemic brain damage in the rat. Annals of neurology, *9*(2), 131-41.

Riezzo, I., Neri, M., De Stefano, F., Fulcheri, E., Ventura, F., Pomara, C., Rabozzi, R., et al. (2010). The timing of perinatal hypoxia/ischemia events in term neonates: a retrospective autopsy study. HSPs, ORP-150 and COX2 are reliable markers to classify acute, perinatal events. *Diagnostic pathology*, *5*, 49.

Romijn HJ, Hofman MA, Gramsbergen A (1991) At what age is the developing cerebral cortex of the rat comparable to that of the full-term newborn human baby? Early Hum Dev 26:61-67.

Rowe JC, Holmes GL, Hafford J, Baboval D, Robinson S, Philipps A, Rosenkrantz T, Raye J (1985) Prognostic value of the electroencephalogram in term and preterm infants following neonatal seizures. Electroencephalogr Clin Neurophysiol 60:183-196.

Sarnat HB, Sarnat MS (1976) Neonatal encephalopathy following fetal distress. A clinical and electroencephalographic study. Arch Neurol 33:696-705.

Scher MS, Beggarly M (1989) Clinical significance of focal periodic discharges in neonates. J Child Neurol 4:175-185.

Scher MS, Alvin J, Gaus L, Minnigh B, Painter MJ (2003) Uncoupling of EEG-clinical neonatal seizures after antiepileptic drug use. Pediatr Neurol 28:277-280.

Selton D, Andre M (1997) Prognosis of hypoxic-ischaemic encephalopathy in full-term newborns--value of neonatal electroencephalography. Neuropediatrics 28:276-280.

Sherman DL, Brambrink AM, Ichord RN, Dasika VK, Koehler RC, Traystman RJ, Hanley DF, Thakor NV (1999) Quantitative EEG during early recovery from hypoxic-ischemic injury in immature piglets: burst occurrence and duration. Clin Electroencephalogr 30:175-183.

Shinnar S, Berg AT, Moshe SL, Petix M, Maytal J, Kang H, Goldensohn ES, Hauser WA (1990) Risk of seizure recurrence following a first unprovoked seizure in childhood: a prospective study. Pediatrics 85:1076-1085.

Sorge, G., & Sorge, A. (2010). Epilepsy and chromosomal abnormalities. *Italian journal of pediatrics*, *36*, 36.

Sperber EF, Haas KZ, Stanton PK, Moshe SL (1991) Resistance of the immature hippocampus to seizure-induced synaptic reorganization. Brain Res Dev Brain Res 60:88-93.

Stafstrom, C. E., Thompson, J. L., & Holmes, G. L. (1992). Kainic acid seizures in the developing brain: status epilepticus and spontaneous recurrent seizures. Brain research. Developmental brain research, *65*(2), 227-36.

Stefovska VG, Uckermann O, Czuczwar M, Smitka M, Czuczwar P, Kis J, Kaindl AM, Turski L, Turski WA, Ikonomidou C (2008) Sedative and anticonvulsant drugs suppress postnatal neurogenesis. Ann Neurol 64:434-445.

Tharp BR, Cukier F, Monod N (1981) The prognostic value of the electroencephalogram in premature infants. Electroencephalogr Clin Neurophysiol 51:219-236.

Tharp BR, Scher MS, Clancy RR (1989) Serial EEGs in normal and abnormal infants with birth weights less than 1200 grams--a prospective study with long term follow-up. Neuropediatrics 20:64-72.

Thoresen M, Penrice J, Lorek A, Cady EB, Wylezinska M, Kirkbride V, Cooper CE, Brown GC, Edwards AD, Wyatt JS, . (1995) Mild hypothermia after severe transient hypoxia-ischemia ameliorates delayed cerebral energy failure in the newborn piglet. Pediatr Res 37:667-670.

Vanhatalo S, Hellstrom-Westas L, de Vries LS (2009) Bumetanide for neonatal seizures: Based on evidence or enthusiasm? Epilepsia 50:1292-1293.

Vannucci RC (1990) Experimental biology of cerebral hypoxia-ischemia: relation to perinatal brain damage. Pediatr Res 27:317-326.

Vannucci RC, Brucklacher RM, Vannucci SJ (1996) The effect of hyperglycemia on cerebral metabolism during hypoxia-ischemia in the immature rat. J Cereb Blood Flow Metab 16:1026-1033.

Vannucci RC, Towfighi J, Vannucci SJ (1998) Hypoxic preconditioning and hypoxic-ischemic brain damage in the immature rat: pathologic and metabolic correlates. J Neurochem 71:1215-1220.

Vannucci RC, Connor JR, Mauger DT, Palmer C, Smith MB, Towfighi J, Vannucci SJ (1999) Rat model of perinatal hypoxic-ischemic brain damage. J Neurosci Res 55:158-163.

Volpe JJ. (2001) Neurology of the newborn. WB Saunders

Walsh BH, Low E, Bogue CO, Murray DM, Boylan GB (2011) Early continuous video electroencephalography in neonatal stroke. Dev Med Child Neurol 53:89-92.

Welsh FA, Vannucci RC, Brierley JB (1982) Columnar alterations of NADH fluorescence during hypoxia-ischemia in immature rat brain. J.Cerebral Blood Flow Metabol 2:221-228

Wasterlain CG (1997) Recurrent seizures in the developing brain are harmful. Epilepsia 38:728-734.

Watanabe T (2003) An automated experimental milker for rat. J Vet Med Sci 65:557-562.

Williams CE, Gunn AJ, Mallard C, Gluckman PD (1992) Outcome after ischemia in the developing sheep brain: an electroencephalographic and histological study. Ann Neurol 31:14-21.

Wirrell EC, Armstrong EA, Osman LD, Yager JY (2001) Prolonged seizures exacerbate perinatal hypoxic-ischemic brain damage. Pediatr Res 50:445-454.

Wusthoff CJ, Dlugos DJ, Gutierrez-Colina A, Wang A, Cook N, Donnelly M, Clancy R, Abend NS (2011) Electrographic seizures during therapeutic hypothermia for neonatal hypoxic-ischemic encephalopathy. J Child Neurol 26:724-728.